DETECTION OF SALMONELLA IN FOOD SAMPLES USING EXOGENOUS VOLATILE ORGANIC COMPOUND METABOLITES

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DETECTION OF SALMONELLA IN FOOD SAMPLES USING EXOGENOUS VOLATILE ORGANIC COMPOUND METABOLITES

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Abstract

Rapid, sensitive and selective detection and identification of pathogens is required in the prevention and recognition of problems related to food security. *Salmonella* is one of the dangerous foodborne pathogens. The identification of specific volatile organic compounds (VOCs) produced by *Salmonella* may contribute in providing a fast and accurate detection method for *Salmonella* in food samples. In this study, VOCs liberated by *Salmonella* strains have been identified and quantified *via* head space-solid phase microextraction coupled to gas chromatography/mass spectrometry (HS-SPME GC/MS). The dominant chemical class of volatiles liberated from *Salmonella* strains was alcohol compounds. In addition, ester and ketone compounds were also detected. The most sensitive VOCs detected were ethyl octanoate (LOD = 62.0 ng/mL and LOQ = 207 ng/mL) and ethyl decanoate (LOD = 66 ng/mL and LOQ = 219 ng/mL) with the lowest LOD and LOQ when using Rappaport-Vassiliadis Soya peptone (RVS) broth media and polar SPME fiber with polar GC column. The type of culture medium was found to affect the liberated VOCs. For example, 2-heptanone was not detected when *S. london* and *S. stanley* were grown in TSB but they were detected and quantified when using BHI as growth media. Also, 1-octanol was detected and quantified in all strains when *Salmonella* grown in TSB and BHI, and did not detected in all strains when RVS was used as growth media.

The research has been extended to include the addition of specific enzyme substrates to the culture medium (RVS). The enzyme substrates are either commercially available or have been synthesised to allow exogenous VOC detection. The specific enzymes targeted in *Salmonella* were α-galactosidase, C-8 esterase and pyrrolidonyl peptidase. The enzyme substrates used are phenyl α-D-galactopyranoside, 2-chlorophenyl octanoate and L-pyrrollidonyl

fluoroanilide respectively. All, except pyrrolidonyl peptidase, are known to give a positive response to *Salmonella*. This developed methodology was initially applied to pure cultures of *S. stanley* to evaluate the feasibility of the approach. The developed approach shows potential for future application in food samples to detect and identify *Salmonella* species in food samples of a level as low as 10^0 CFU /mL within a 5 h incubation at 37 ºC by the detection of the liberated VOCs.

Subsequently the methodology was applied to a range of food samples (milk, cheese, eggs and chicken). It was found that all food samples were *Salmonella* free; however, false positive was detected due to the presence of other pathogens in the food samples. Inhibition of some of these pathogens in milk and cheese samples was achieved with the addition of 5 mg / L vancomycin and 10 mg / L of novobiocin. To improve the method specificity, it was necessary to deviate from the standard method and use *Salmonella* selective RVS broth in pre-enrichment step than using non selective one (BPW). This results in a successful detection of *Salmonella* contamination on milk samples and cheddar cheese samples. However, failed in detect *Salmonella* in other cheeses. Inhibition of resistant pathogens (*Streptococcus salivarius ssp. Thermophilus*, *Lactobacillus rhamnosus* and *Enterococcus faecalis*) using another combination of selective agents (vancomycin 10 mg /L, novobiocin 10 mg /L, erythromycin 0.75 mg /L and lithium chloride 15 g/ L) failed.

This study highlighted the benefits of the use of specific enzyme substrates along with antibiotics into *Salmonella* VOC analysis to improve the specificity of *Salmonella* detection method. The results of VOC analysis of specific enzymes inherent within *Salmonella* could be extended to develop a selective portable sensor approach to be used in food production.

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List of Abbreviations and Symbols Used

4-methylumbelliferyl	MUF
Acidity	pH
Alpha	α
American Type Culture Collection	ATCC
Ammonium ion	NH_4^+
Aromatic hydrogen	Ar*H*
Beta	β
biogenic amines	BA
Brain Heart Infusion	BHI
Buffered Peptone Water	BPW
C-8 esterase	C-8 E
Carbon Nuclear Magnetic Resonance Spectroscopy	^{13}C-NMR
Colony Forming Units	CFU
Correlation coefficients	R^2
Coupling constant	J
Cystine lactose electrolyte deficient medium	CLED
Degree Celsius	º C
Delta (chemical shift scales)	δ
Deoxyribonucleic acid	DNA
Deuterated chloroform	$CDCl_3$
Deutsche Sammlung von Mikroorganismen	DSM
Dichloromethane	DCM
Dimethylformamide	DMF
Doublet	d
Electron volt (s)	eV
electron-ionization	EI
European Union	EU
Food and Drug Administration	FDA
Food Emergency Response Network	FERN

Gas chromatography mass spectrometry	GC/MS
Gram	g
Headspace	HS
Hertz (unit of frequency)	Hz
High Resolution Mass spectrometry	HRMS
Hour (s)	h
Hydrochloric acid	HCl
Immunomagnetic separation	IMS
Enzyme-linked Immunosorbent Assay	ELISA
International Organization for Standardization	ISO
Ion mobility spectrometry	IMS
Isobutyl chloroformate	IBCF
Kilo Volt (unit of voltage)	kV
Kilopascal	kPa
Limit of detection	LOD
Limite of Quantification	LOQ
liquid chromatography mass spectrometry	LC-MS
Litre (s)	L
Magnesium sulphate	$MgSO_4$
Mass to charge ratio	m/z
Matrix-Assisted Laser Desorption/Ionization-	
Mega Hertz	MHz
Micro gram	µg
Microliter	µL
Milimol	mmol
Milligram	mg
Millilitre	mL
Millimeter of mercury	mmHg
Minimum inhibitory concentration	MIC
Minute (s)	min
Molarity	M
Molecular ion	M+

Multiplet	m
Nanogram	ng
N-Methyl-2-pyrrolidone	NMP
N-methylmorpholine	NMM
Non-Typhoidal *Salmonella*	NTS
Normality	N
peptide mass fingerprint	PMF
Polyacrylate	PA
Polydimethylsiloxane	PDMS
Polymerase chain reaction	PCR
Polytetrafluoroethylene	PTFE
Potasium hydroxide	KOH
Principal Component Analysis	PCA
Proton Nuclear Magnetic Resonance Spectroscopy	^{1}H-NMR
Pyrrolidonyl peptidase	PYRase
Quartert	q
Rappaport-Vassiliadis Soya peptone broth	RVS
Retention time	t_R
Salmonella identification medium	SM-ID
Salmonella selective medium	ABC
Singlet	s
Sodium hydroxide	NaOH
Solid Phase Microextraction	SPME
Solid phase microextraction-electrodeposition	SPME-ED
Standard Deviation	SD
Standard Plate Counts	SPCs
The Centers for Disease Control and Prevention	CDC
The National Collection of Type Cultures	NCTC
Trifluoroacetylacetone	TFAA
Time of Flight-Mass Spectrometry	TOF-MS
Triethylamine	Et_3N
Trifluoroethyl octanoate	TFEO

Triplet	t
Tryptone soya agar	TSA
Tryptone Soya broth	TSB
Volatile Organic Compound	VOC
α-Cyano-4-hydroxycinnamic acid	HCCA
o-Nitrophenyl-β-D-galactopyranoside	ONPG

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Declaration

I declare that this thesis contained the results of the research work undertaken by me in the Faculty of Health and Life Sciences under the supervision of Professor John R. Dean, Professor Stephen Stanforth and Professor John David Perry between February 2013 and January 2017. This thesis has not been previously submitted in part or whole for any other award of degree.

Name:

Signature:

Date:

Chapter 1: Introduction

1.1 Identification/detection of *Salmonella*

This study is an investigation into the very important topic in food safety worldwide. Detection of pathogenic bacteria is important to protect consumers and prevent human foodborne illness and for effective treatment of patients as well as to reduce high medical and economical costs. The most important goal of this study was to develop accurate, rapid and sensitive analytical detection technique for the dangerous pathogenic bacteria *Salmonella*.

Salmonella is bacteria that is considered as the most common causes of food poisoning and is the most important cause of food-borne bacterial illnesses in animal and humans. The pathogenic *Salmonella* is a life-threatening bacterium can cause more serious illness in older adults, infants, and persons with chronic diseases and causes high mortality rates (Buckle *et al*., 2012).

Salmonella, a genus within Enterobacteriaceae, are Gram-negative rod-shaped bacteria (Su and Chiu, 2007). The genus *Salmonella* includes only two species,*Salmonella enterica* and *Salmonella. bongori*. The type species *Salmonella enterica* is divided into six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae and indica*) and most pathogenic *Salmonella* belong to the subspecies *Salmonella enterica* subsp. *enterica* (Tindall *et al*., 2005; Su and Chiu, 2007). There are more than 2600 different serovars of *Salmonella enterica* and they are differentiated by their antigenic presentation and can be divided into typhoidal and non-typhoidal *Salmonella* (NTS) based on the type of infection causes (Gal-Mor *et al*., 2014). Infections with *Salmonella* are started when the pathogen the *Salmonella* typhi bacteria attacks the gastrointestinal epithelium

Typhoidal *Salmonella* serovars belong to *Salmonella* typhi and *Salmonella* paratyphi. Illness associated with fever caused by these serovars is called enteric

fever, also known as, typhoid or paratyphoid fever. Enteric fever is an invasive, life-threatening, disease. The estimated global annual reported cases were over 27 million cases, in which more than 200,000 cases were as deaths cases (Crump *et al*., 2004; Buckle *et al*., 2012). The Non-Typhoidal *Salmonella* (NTS) salmonelloses occur globally. There are an estimated 93.8 million cases of gastroenteritis due to NTS infection each year, and there are approximately 155,000 deaths among these cases (Majowicz *et al*., 2010).

Added to the importance of this study, *Salmonella* can be associated with many kinds of foods and cause the spread of outbreaks worldwide (Carrasco *et al*., 2012). *Salmonella* is widely spread in nature and often lives in the gut of many farm animals. Therefore, *Salmonella* transmitted to humans through consumption of contaminated food of animal origin, such as meat, poultry, eggs and milk. In addition to contaminated animal-derived food products, transmission of *Salmonella* can result from person to person contact or from contact with pets such as cats, dogs, rodents, reptiles, or amphibians (Hilbert *et al*., 2012; Haeusler and Curtis, 2013). Another important source of infection with *Salmonella* is consumption of contaminated vegetables, sprouts, tomatoes, fruits, peanuts, and spinach which have all been associated with recent outbreaks (Brandl, 2006; Lapidotand and Yaron, 2009; Jackson *et al*., 2013; Bayer *et al*., 2014).

It is important to determine the source of contamination to reduce the risk of infection and also for rapid patient treatment with the right antibiotics. Severe regulatory actions have been taken in order to improve the food safety practices however, there is still a need for enhanced rapid tools for food pathogen detection. Looking into the literature, much work has been done to overcome the problem by developing pathogen identification methods. Starting from the traditional detection method which is, a time-consuming detection method of *Salmonella*

spp. from food samples. The development of the traditional method includes isolation of *Salmonella* using a multistep protocol with nonselective pre-enrichment, followed by a selective enrichment step, isolation on selective agar media and a preliminary biochemical and serological confirmation. Numerous culture protocols have been developed and modified to reliably recover and characterize *Salmonella* species from a broad range of sources including food. However, the culture method is time consuming and cannot monitor foodstuffs effectively, especially the very short shelf life foods also it is not effective for patient treatment (Bell *et al*., 2016). Therefore, the speed of detection of this pathogen have greatly improved using other methodologies including immunology-based techniques, nucleic acid-based techniques and diagnostic biosensors.

The rapid methods have been developed to speed up the detection and also to have high sensitivity that are enough to detect one cell in a studied sample as the infective dose of *Salmonella* is very low. Nevertheless, food samples possess a distinct set of challenges for these rapid methodologies as the identification strategy still relies heavily on the availability of a pure isolate. However, headspace VOCs analysis techniques are meeting this challenge by advancing the detection and identification of pathogens with incorporation into growth media enzyme substrates that will liberate exogenous VOC biomarkers upon enzymatic metabolism. Among these techniques capable of determining VOCs, the highly sensitive HS-SPME-GC/MS technique is the most suitable one. It is a well establish technique which allows direct separation and identification of culture VOCs (Tait, 2012; Tait *et al*., 2014a, b).

The major criterion for evaluating an identification procedure must be accuracy of identification. Therefore, in order to identify *Salmonella* in food

samples with a high degree of specificity in terms of key VOCs it is necessary to determine the enzyme activity exhibited by *Salmonella* to modify the growth medium by the addition of a desired substrate that liberates a unique VOC. Some commercial enzyme substrates are labelled with volatile compounds, and most enzyme substrates that are available to purchase incorporate compounds that are chromogenic or fluorogenic. There is a need to synthesis enzyme substrates, that are tagged with un-natural volatile compounds. One of the commercially available substrates used in biochemical assays are labelled with volatile compounds such as phenol, for example, phenyl α-D-galactopyranoside which is hydrolysed by the enzyme α-galactosidase. Detection of phenol in the headspace provides a significant advantage as there is no potential for interference in the measurement by the assay medium. The chosen enzymes must be specifically produced by *Salmonella*. However, it is possible for food samples to contain other α -galactosidase producing bacteria. It is therefore important to use a group or number of enzymes to aid reliable identification of *Salmonella* and avoid the detection of false positive results. Then, for accurate identification enzymes such as C-8 esterase, pyrrolidonyl peptidase (PYRase) and decarboxylases can be used.

Bad outcome, high hospitalization costs, food recall and food industry concerns are the consequences of the currently existing limited pathogen identification possibilities. For these reasons, we aimed to apply the innovative method of HS-SPME GC/MS as a potential reliable identification of *Salmonella* by detection of volatile metabolomes in food samples include milk, cheese, eggs and chicken.

Milk and dairy foods are good sources of calcium, vitamin D, protein and other essential nutrients. Raw milk or cheese made from raw milk can carry

harmful bacteria such as *Salmonella* and by consumption of such food it is possible to get risk of foodborne illnesses. Pasteurisation is the established method of lowering microbial numbers and eliminating pathogen from milk to make it safe for consumption. *Salmonellae* are not heat resistant which means they can be readily destroyed at pasteurization method. However, Salmonellosis has been reported due to consumption of pasteurised milk (Ryan *et al*., 1987; Ackers *et al*., 2000). Thus also pasteurised milk has a potential to transfer *Salmonella* from infected farm animals to humans (Olsen *et al*., 2004; Mazumdar *et al*., 2007). Therefore, there is a need to develop rapid, sensitive and specific methodologies to detect *Salmonella* in milk and cheese.

Eggs are among the most nutritious foods with several health benefits (Ruxton *et al*., 2010). However, according to a number of studies (Haglund *et al*., 1964; Greenfield *et al*., 1971; Duguid and North, 1991; Guard-Petter, 2001; Ohtsuka *et al*., 2005; Malorny *et al*., 2007; Foley *et al*., 2011) eggs and eggs product are contaminated with *Salmonella* which may detract from the beneficial effects of eggs consumption. Poultry meat is considered healthy food and is one of the most popular food which contains high level of protein and low fat content with unsaturated fatty acids. Poultry products have always topped the incidence of salmonellosis in many countries (Jørgensen *et al*., 2002; Orji *et al*., 2005; El-Aziz, 2013). Contamination with *Salmonella* in poultry products can occur at multiple steps along the food chain, which includes production, processing, distribution, retail marketing, handling and preparation (Jørgensen *et al*., 2002; Dookeran *et al*., 2012).

The poultry meat, eggs, milk and cheese are vehicles of *Salmonella* transfer and play important roles in disease prevalence. Reducing the risk of *Salmonella* contamination and identifying accurately the contamination sources

in these foods is needed. In spite of continuing research efforts, well-timed and simple pathogen detection with a high degree of sensitivity and specificity remains an elusive goal and much interest to us.

1.2 Objectives

The overall objective of this thesis was to develop a rapid, sensitive and accurate detection method for *Salmonella* in food samples. It is apparent from the above discussions that several steps need to be carried out to attain this objective.

The first step will be to investigate the volatile compounds liberated by *Salmonella* in the headspace of inoculated sterilized broth samples when incubated overnight at 37°C. The liberated VOCs will be extracted and concentrated using HS-SPME, this will be combined with GC-MS for separation and identification of the generated VOCs. Six strains of *Salmonella* inoculated in sterile BHI, TSB and RVS broths will be examined for this purpose. The liberated VOCs will be identified by comparing their retention times and mass spectra with authentic standards and quantified using an external calibration method. It is imagined that the *Salmonella* VOC profiles will act as marker, aiding *Salmonella* identification therefore, the type of growth media and metabolic capabilities of the strains and the polarity of the GC column will be tested for their influence on the detected *Salmonella* VOC profiles.

An accurate identification method for *Salmonella* needs to be developed by screening and monitoring VOCs generated by *Salmonella* during hydrolysis of specific enzyme substrates. Sufficient number and type of enzymes of *Salmonella* to develop unambiguous identification in food samples will be examined. Namely α-galactosidase, C-8 esterase, pyrrolidonyl peptidase (PYRase) and ornithine and lysine decarboxylase. Several commercial and

synthesised enzyme substrates will be tested. The structures of the synthesised substrates will be investigated by NMR experiments before being applied to the *Salmonella* experiments. The *Salmonella* investigations will focus first on testing the six strains on pure cultures in liquid media without addition of food. The strain which generates the highest concentration of the VOCs will be selected for all food experiments. Ready-to-eat foods should be free of *Salmonella* therefore, the sensitivity of the method will be assessed in terms of initial inoculum size. In addition, an investigation to how accelerate the analysis and the detection will be carried out.

Variety of food types considered as the most common sources of *Salmonella* will be tested with the developed detection method. These foods include milk, cheese, eggs and chicken. The HS-SPME-GC/MS and MALDI-TOF-MS will be used to analyse the food samples for generated VOCs and identify the present pathogens, respectively. The method will be evaluated for its specificity and accuracy using some selective agents. In further attempts to increase the specificity of the method a modification step to the standard method will be carried out.

The goal of this study was not just to develop an analytical chemistry method to detect *Salmonella* in food samples. When this project started the goal was also to simplify the proposed analytical method and develop an alternative approach that did not require analytical instrumentation and was easy to use in the food industry. Developing a colorimetric method based on the reaction of the liberated VOCs with a reagent and develop colour that can be optically detected by either the naked eye or colorimetric analysis. However, because of the limited frame time this goal could not be achieved.

A detailed literature survey has been carried out in this thesis covering various aspects of *Salmonella* as a foodborne pathogen and is presented in Chapter 2. Chapter 3 deals with all experimental work done in relation to this thesis. Results and discussions are presented in chapters 4. 5, 6, 7, and 8. Conclusions and recommendations in Chapter 9. The thesis will end up with chapter 10 a list of the references used.

Chapter 2: Literature survey

2.1 Introduction

The objective of this chapter is to develop an understanding of some food microbiology aspects and analytical information needed to undertake research developing an analytical method for pathogen detection. A description of *Salmonella* and *Salmonella* contamination in food and its health consequence is provided. Various methods for *Salmonella* detection are discussed in detail in this chapter. Literature on different techniques used to detect *Salmonella* in food samples are discussed with a focus on the analytical approach for VOC detection as a suitable technique. Literature on *Salmonella* key enzymes targeted in this study are reviewed.

2.2 Food safety and bacterial contamination

Food is a requirement for life. To meet this requirement, food production systems are increasing in complexity and size throughout the world. When we select and consume food there is an expectation to be safe to eat. This expectation places essential responsibilities on the people who work with food to make a confidence of the absence of any risk of harm from food. Therefore, food safety has become a major concern of both governments and consumers in the past and recent years (Phillips, 1998; Van Boxstael *et al*., 2013). The hazard may be physical, chemical or microbial including bacteria. It is well known that food contains lots of bacteria which enter foods from both internal and external sources. Bacteria have both desirable and undesirable roles in our food some are useful and safe for health however, some are not safe and dangerous. The two categories of bacteria that create major problems in the food industry are spoilage bacteria and pathogenic bacteria. Spoilage bacteria break down protein, causing spoilage or putrefaction which may be detectable by smell, but do not

usually cause food poisoning (Chen *et al*., 2014). Whereas pathogenic bacteria are responsible for causing illness whether they are present in small or large numbers in food. They do not alert the appearance, taste or smell of food. Food can become contaminated with pathogenic bacteria at any stage during its production, processing, storing or cooking. The health consequences of pathogen contamination are food–borne illness that includes food poisoning and food-borne disease.

Food poisoning can be by ingestion of food containing large numbers of bacteria or the harmful compounds (toxins) produced by these bacteria when grown in food and consequently the symptoms come on very quickly. Whereas, when ingestion of food contains relatively small numbers of viable bacteria the illness is delayed. This is because bacteria need time to multiply in the intestine before stick to the lining of the intestine and destroying those cells or produce toxins (poisons) (Jones *et al*., 1994). The appearance of the symptoms depends on the type of bacteria and how many are swallowed. It could be hours or days.

As the bacteria enter the body through the digestive system the symptoms will generally be in this part of the body. These symptoms include nausea, vomiting, abdominal cramps and diarrhoea. In some cases, contaminated food can cause very serious illness or even death. Scallan *et al*. (2011) estimated that each year 31 major pathogens are acquired in the United States (Scallan *et al*., 2011). The 5 most dangerous foodborne pathogens according to Food Safety News are *Listeria, Salmonella*, *Escherichia coli (E. coli)*, *Vibrio vulnificus* and *Clostridium botulinum* (The 5 most dangerous foodborne pathogens, 2016). In the European Union (EU) *Salmonella* is reported to be the second cause of foodborne disease after *Campylobacter* with 88,715 confirmed cases in 2014 followed by *Listeria* (Wood, 2016).

2.3 *Salmonella*

Salmonella is a genus of rod-shaped Gram negative bacteria (Figure 2.1) that belong to the family *Enterobacteriaceae*. Their species are motile, oxidase-negative, catalase positive and utilize glucose and other carbohydrates with the production of acid and gas (Srebernich *et al*., 2011).

Figure 2.1 A culture of *Salmonella* bacteria on a plane surface (ttp://www.alamy.com)

Salmonella is named after an American bacteriologist, D. E. Salmon, who first isolated *Salmonella choleraesuis* from porcine intestine in 1884 (Su and Chiu, 2007). Officially the genus of *Salmonella* contains two species*, S. enterica*, and *S. bongori. S. enterica* consists of six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. salamae; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *Indica* which are then further subdivided into serotypes (Su and Chiu, 2007). In subspecies I, (S. *enterica* subsp. *enterica*) the serotypes are given specific names either according to the disease and/or the animal from which the organism was isolated or usual habitats, such as *S. typhi* and *S. typhimurium*, or by the geographical area where the strain was first isolated, e.g., *S. London* and *S. panama* (Su and Chiu, 2007).

There are more than 2,600 serotypes of *Salmonella* differentiated by their antigenic presentation (Gal-Mor *et al*., 2014). More than 50% of these serotypes belong to the *Salmonella enterica* subsp. *enterica* (Srebernich *et al*., 2011). *S. enterica* has adapted more than *S. bongori* to live in the intestine of man and warm-blooded animals, whereas S. bongori travels in the external environment and is detectable in the intestinal contents of warm-blooded animals, so it is rare for it to be found in food for human consumption (Giaccone *et al*., 2011). Therefore, the strains most frequently involved in human disease are *S. enterica* subsp. *enterica*. They are responsible for 99% of human salmonellosis (Srebernich *et al*., 2011). As the enteric means pertaining to the intestine, this means *Salmonella* strains can survive and multiply in the gastrointestinal (GI) tract of humans and animals. The *Salmonella enterica* are involved in causing diseases of the intestines and the three main serovars of *Salmonella enterica* are *Typhimurium*, *Enteritidis*, and *Typhi.*

Most *Salmonella* serotypes can grow over the temperature range of 6 – 48 ºC with an optimum temperature in the range of 32 - 37°C, therefore the majority of *Salmonella* serotypes are not particularly heat resistant and can be readily destroyed at the pasteurization temperature (71.7 ºC for 15 seconds) (Phillips, 1998; Barbara *et al*., 2000; Odumeru and León-Velarde, 2011). The optimum pH for *Salmonella* growth is 6.5 – 7.5 but a few *Salmonella* serotypes can grow over a range of pH values from 4 - 9.5 (Lawley, 2013) however, they are killed by acid below pH 4 (Phillips, 1998). *Salmonella* is quite resistant to adverse conditions (Spector and Kenyon, 2012) and this allows them to persist in the environment and spread along the food chain, from the animals to the food of animal origin, or to plants that are fertilized with animal manure. For example, *Salmonella* are not able to grow in dry environments however, may survive for some time on dry food

production surfaces also, all *Salmonella* strains can grow with or without oxygen (facultative anaerobes) and in atmospheres containing high levels of carbon dioxide (up to 80 %) (Lawley, 2013).

2.4 Associated Foods

Salmonella spp. are the most common pathogenic bacteria associated with a variety of foods. These days *Salmonella* cause many foodborne disease outbreaks where most of the earlier and recent food products recalls are due to *Salmonella* (Food Poisoning, 2016). In addition, *Salmonella* is one of the most studied pathogens and accounts for 31 % of 90 % estimated food-related deaths (Mead *et al*., 1999). As *Salmonella* live in the gut of many farm animals *Salmonella* transferred to humans through consumption of contaminated food of animal origin such as; meat, poultry, eggs, milk and dairy products (Sanchez *et al*., 2002). The most contaminated foods are poultry, eggs and dairy products. These foods are almost certainly the most common cause of human Salmonellosis worldwide (Herikstad *et al*., 2002). As *Salmonella* is also found in water and soil other foods like green vegetables, fruit herbs, spices and sprouts can become contaminated and cause human illness (Brandl, 2006; Lapidotand and Yaron, 2009; Jackson *et al*., 2013; Bayer *et al*., 2014). Furthermore, *Salmonella* may be found in cooked ready to eat food due to insufficient cooking of contaminated foods, or from cross contamination from raw food e.g. raw poultry, to cooked foods. Also, the use of raw egg in dishes can lead to infection with *Salmonella*, also contamination with *Salmonell*a could be due to poor personal hygiene (Carrasco *et al*., 2012). In addition, pets, insects, birds and flies may also transmit *Salmonella* to different foods (Hilbert *et al*., 2012).

Moreover, Salmonellosis have been reported due to consumption of pasteurised milk (Ryan *et al*., 1987; Ackers *et al*., 2000; Olsen *et al*., 2004;

Mazumdar *et al*., 2007). The pasteurization process is effective at removing *Salmonella*. However, pasteurised milk has a potential to transfer *Salmonella* from infected farm animals to humans due to the occurrence of poor sterility or improper pasteurization. The milk can be also contaminated by unsanitary handling after the completion of the pasteurization process.

2.5 *Salmonella* the food-borne disease

The dangers for human health mainly arise from food. Consumption of contaminated water or food is the main source of bacterial infection and diseases in human. *Salmonella* have been recognised as the cause of enteric disease for many years. The infection by *Salmonella* is called salmonellosis. Many cases of salmonellosis, occur worldwide every year and the disease results in more than a hundred thousand deaths (WHO, 2013; Gal-Mor *et al*., 2014). The Centers for Disease Control and Prevention (CDC) estimated *Salmonella* to be the second of eight known pathogens causing foodborne illnesses, and the first pathogen causing hospitalizations and deaths in the United States (Burden of Foodborne Illness, 2016). In the UK according to Public Health England reports, there was 799 serotypes of *Salmonella* infections recorded in October 2016 (*Salmonella* infections faecal specimens in England and Wales, 2016).

Salmonella strains cause very different diseases and distinct immune responses in humans (Gal-Mor *et al*., 2014). According to the type of diseases caused *Salmonella* strains can be divided into typhoidal and non-typhoidal *Salmonella* (NTS) serovars. Typhoid fever, caused by *S. enterica* serotype *S. Typhi* and *S. Paratyphi* A. It is a bacteremic illness which clinically differ from other Gram-negative bacteremias. The non-typhoidal *Salmonella* (NTS) serotypes cause self-limiting diarrhoea with occasional secondary bacteremia (De Jong *et al*., 2012).

Generally, symptoms of *Salmonella* infection, or Salmonellosis, range widely. The most common symptoms include vomiting, diarrhoea, abdominal cramps, and fever. Other symptoms may also occur such as, headaches and loss of weight and appetite. The incubation period is 5-72 hours but may be as long as seven days with symptoms occurring between 12 and 36 hours after infection and lasting two to five days (Phillips, 1998). The NTS cause moderately serious gastroenteritis diseases with a quick recovery and usually without the need to resort to specific therapies. In some *Salmonella* infection cases where the elderly or very young, or immunocompromised persons are affected salmonellosis may also lead to the patient's death (*Salmonella* warning after food poisoning death, 2016). In addition, the severity of the illness depends on the type of the food and the number of cells ingested. The infected dose is usually one million cells but if the food vehicle contains a high fat content such as chocolate or cheese then it may be as low as ten cells particularly if susceptible individuals are involved (Phillips, 1998; Alberts, 2002). This is because it has been suggested that the high fat content somehow protects the organism from the acid stomach environment allowing a more efficient colonisation of the intestinal mucosa (Phillips, 1998; Waterman and Small, 1998; Spector and Kenyon, 2012).

2.5.1 *Salmonella* serotypes and foodborne illnesses

Almost all *Salmonella* enteric strains are able to be transmitted not only by animal-derived foods but also by plant products and are able to cause human disease. In Food Safety News, Robinson (2013) reported the five most common serotypes of *Salmonella* causing foodborne illnesses. First the most common strain of *Salmonella* in the food supply *is Salmonella Enteritidis*. This serotype is most often associated with poultry. Also, *S. pullorum* and *S. gallinarum* were

widespread in poultry however, they were minimized through aggressive suppression programs. The second most common serotype associated with foodborne illness is *S. typhimurium* and the third most frequently identified with chicken. *S. typhimurium* is also linked to ground beef, pork and other poultry products (Bosilevac *et al*., 2009; Techathuvanan *et al*., 2010; El-Aziz, 2013). This strain has known to be antibiotic-resistant, which lead to eliminating the pathogen from food products very challenging (Su *et al*., 2004; Bosilevac *et al*., 2009).

The third most common *Salmonella* serotype associated with foodborne illness is *S. Newport*. This strain is most often found in turkey products, cantaloupe, live poultry and alfalfa sprouts and also has proven to be antibiotic-resistant (Lynne *et al*., 2008; Sangal *et al*., 2010; Van Beneden *et al*., 1999). The fourth most common *Salmonella* serotype associated with foodborne illness is S. *Javiana* which has been linked to contaminated mozzarella cheese, watermelon, bass, poultry, lettuce and tomatoes (Hedberg *et al*., 1992; Blostein, 1993; Guo *et al*., 2001; Control and Prevention, 2005). The fifth most common *Salmonella* serotype associated with foodborne illness and the second most frequently associated with human health issues is *S. Heidelberg* (Robinson, 2013).

2.6 Detection of pathogens in Food

The importance in determining the source of contamination is to reduce the risk of infection and also for effective patient treatment. The methods used for the microbiological evaluation in foods are quantitative and qualitative methods. Quantitative methods are designed to estimate directly or indirectly the microbial load in a studied sample. Examples of some of the quantitative methods used are aerobic plate counts (APCs), or standard plate counts (SPCs). In contrast, qualitative methods are designed to determine whether a sample of a food contains a specific microbial species among the total microbial population.

Such methods can be used to detect the possible presence of *Salmonella* in food samples.

Although food safety practices are being improved due to severe regulatory actions. For example, due to continued improvement of egg and poultry hygiene the number of laboratory-confirmed cases of salmonellosis across the UK in 2013 was 8,924 compared with 9,307 in 2012 (Food Standards Agency, 2014). As recently still there have been many outbreaks and product recalls due to *Salmonella* contamination there is still a need for enhanced rapid tools for food pathogen detection (Outbreak Investigation, 2016). An example, establishing the difficulties in promptly detecting contaminations and avoiding their spread is the recent outbreak of *Salmonella* in England which was in September 2016, when an unusual strain of *Salmonella enteritidis* PT 14b, was found in eggs and made more than 150 people ill and one person died in Cheshire (*Salmonella* warning after food poisoning death, 2016).

Salmonella is a potentially life threatening bacteria. Even small numbers of viable cells of *Salmonella* present in food have the potential to establish and multiply in the digestive tract and cause Salmonellosis. To avoid this threat, the analysis of food products for the presence of this pathogen is one of the basic steps needed. Therefore, efforts have been made to develop and improve *Salmonella* detection methods which have sensitivity enough to detect one cell in the studied samples.

2.7 *Salmonella* detection methods

Generally, *Salmonella* detection methods can be categorized into two groups, conventional *Salmonella* detection methods and rapid *Salmonella* detection methods. Based on the principle applied *Salmonella* detection method can be categorized into several groups, these include conventional culture

methods, immunology-based assays, nucleic acid-based assays, miniaturized biochemical assays, and biosensors (Lee *et al*., 2015). The time required for the conventional analysis and rapid analysis methods depends on the cell enrichment steps to reach minimal cell concentration enough for *Salmonella* detection. The cell enrichment process in a conventional method is longer than the rapid detection method. As the rapid method usually requires at least 10^4 CFU/mL of *Salmonella* concentration for detection (Lee *et al*. 2015). Therefore, the conventional detection methods take a relatively long time and are labour intensive. The rapid methods, many of which are automated, also are quite specific, sensitive and relatively accurate. Numerous researchers have published summaries and reviews for different methods used to detect *Salmonella* in food samples (Carrique-Mas and Davies, 2008; Odumeru and León-Velarde, 2011; Zadernowska and Chajęcka, 2012; Cox Jr *et al*., 2014; Lee *et al*. 2015). The principles and the procedures of some of these methods are briefly presented.

2.7.1 Conventional *Salmonella* detection methods

Culturing can be invaluable for identifying and classifying bacteria since the colonies of particular species often exhibit a particular form of growth. Bacteria grown in nutrient broth and on nutrient agar plates media can exhibit visible physical differences in appearance in their isolated colonies. These differences are called cultural characteristics or morphology and can be used as a means of recognition. These characteristics include: colony size, colour and shape.

Traditional cultural methods are used widely for detection of pathogens include *Salmonella*. The main objective of this method is to determine whether a sample contains viable cells of the contaminated pathogen. For detection of *Salmonella* the cultural methods are established using nutrient acquisition,

biochemical characteristics, and metabolic products unique to *Salmonella spp*. (Ricke *et al*., 1998). To detect/identify *Salmonella* in food samples the species need to be isolated selectively as possible from the sample. Therefore, the isolation procedure contains several steps, such as nonselective pre-enrichment of a defined weight or volume of the food sample, followed by a selective enrichment, and then testing on an agar medium usually by plating onto selective agars, and biochemical and serological confirmation of suspect colonies.

This detection method depends on the use of appropriate media which containing selective and differential agents. Most commonly used media in pre-enrichment step are buffered peptone water (BPW) and lactose broth (Lee, 2015). The enrichment (selective) media have been evaluated and developed to increase the sensitivity and the specificity of *Salmonella* detection. This is done by addition of two or more inhibitory reagents such as bile salts, brilliant green, thiosulphate, deoxycholate, malachite green, novobiocin, tetrathionate, cycloheximide, nitrofurantoin, and sulphacetamide (Schothorst and Renaud, 1985; Lee, 2015). The job of these inhibitors in a selective media is to suppressing bacteria present in the sample and allows continuous growth of *Salmonella* (Tietjen and Fung, 1995). Rappaport-Vassiliadis (RV) medium and tetrathionate (TT) broth has been used as official *Salmonella* enrichment media in approved standard methods such as FDA Bacteriological Analytical Manual (BAM) and FERN *Salmonella* methods (Lee, 2015). Plating media used for *Salmonella* isolation also have been developed and improved gradually. *Salmonella*-Shigella agar (SS), brilliant green agar (BGA), bismuth-sulfite agar (BSA), Hektoen enteric (HE), and xylose-lysine-deoxycholate agar (XLD) are the first frequently used plating media for isolation of *Salmonella*. However, due to some serotypes not being distinctive and even missed on those media, yielding

false negatives and increasing cost for additional tests (Carrique-Mas and Davies, 2008), and also presumptive *Salmonella* colonies isolation, resulting in false positives (Naravaneni and Jamil, 2005) chromogenic and fluorogenic media have been developed to improve the detection of *Salmonella*. These include SM-ID agar, Rambach agar, ABC Medium and BBL CHROM agar *Salmonella*. The use of these media directly on the isolation plate for detection, enumeration, and identification of *Salmonella* made improvement to the conventional methods as these media have been shown to be convenient, reliable, and more specific and selective than conventional media (Perry *et al*, 1999; Alakomi and Saarela, 2009; Perry and Freydiere, 2007; Lee *et al*, 2015).

Different approaches of *Salmonella* enrichment using the unique biochemical physical properties of the organisms have been standardized by several regulatory agencies, for example; International Organization for Standardization (ISO). The ISO method (ISO 6579:2002), consists of a pre-enrichment of samples (25 g) in 225 mL BPW and incubated (24 h at 37-42 ºC) for the injured cells to repair and then multiply in order to reach moderately high numbers (along with many other associated microorganisms). Following transfer an aliquot of the sample from the pre-enrichment broth, is subjected to a selective enrichment in Rappaport-Vassiliadis (soya base) (RVS) broth and incubated (24 h at 37-42 ºC). During incubation time *Salmonella* species are expected to selectively grow to a high number and the associated microorganisms are expected to not grow. A small amount (0.01 mL) of the enrichment broth is then streaked on the surface of a pre-poured selective-differential agar medium plate, which is then incubated for the colonies to develop. From the differential colony characteristics, the presence of *Salmonella* can be tentatively established and

then the cells are purified and examined by the recommended methods for confirmation test for presence of *Salmonella* (Ray, 2005).

Isolation of *Salmonella* using the conventional methods needs to prepare multiple subcultures required for several identification steps, taking more than 5 days for complete isolation and confirmation (Ray 2005; Lee 2015). However, the use of selective media, modified or adapted conventional procedures, eliminating the use of subculture media and further biochemical tests can provide test results 1 day earlier, compared with conventional methods (Eijkelkamp *et al*., 2009). However, these approches are not fast enough for the purpose of screening food samples for *Salmonella*. Therefore, an alternative to the time-consuming culture method is required. Several approaches have been developed to accelerate detection of *Salmonella* in a sample.

2.7.2 Rapid *Salmonella* detection methods

The rapid method may be defined as a method that able to detect *Salmonella* spp. in samples and delivers reliable results within a few hours to a day (Lee, 2015). To overcome the competing flora in food samples and reduce the interference of the food matrix and increase the sensitivity of the detection method there has been always a lot of interest in the development of separation and concentration techniques prior to detection of *Salmonella* in food samples. On that basis, several rapid and automated methods have been developed and used for detection of *Salmonella spp*. in a variety of sample matrices. Generally the rapid test protocols include a selective enrichment stage, and then apply concentration and/or rapid detection techniques to replace culture on selective agars and further confirmatory tests. The rapid detection techniques can be divided into three categories based on the principle used. Immunology-based technique, nucleic acid-based technique and diagnostic biosensors.

Immunoassays include immunofluorescence, immunoimmobilization, enzyme-linked immunosorbent assay (ELISA) and Immunomagnetic separation (IMS) methods. The nucleic acid-based detection methods are genetic methods that include hybridization and the most popular method is the polymerase chain reaction (PCR) technique. PCR is a molecular genetic technique for making multiple copies of a gene, and is also part of the gene sequencing process. Eijkelkamp *et al*. (2009), Odumeru and Lee *et al*. (2015) described the theory, the basis, and the application of these rapid methods for detecting *Salmonella* in food samples providing information about their sensitivity and specificity. For example, the ELISA and PCR procedures show comparable specificity and sensitivity to conventional methods where ELISA assays are able to detect *Salmonella* concentration at the level of 10^4-10^5 CFU / mL while PCR-based assays provide the level of sensitivity of 10^4 CFU / mL after enrichment (Lee *et al*., 2015). The major disadvantage of all immunoassays is the difficulty of getting good quality antibodies, as the accuracy of the entire reaction process depends upon the binding specificity of the antibody to all *Salmonella* cells. This is critical to prevent false-negative results as all *Salmonella* strains have the ability to cause disease in humans, leaving holes in this method if it is used to screen the food supply (Bell *et al*., 2016).

The development and advancement of the PCR technique improves the specificity and sensitivity for detecting *Salmonella* in very low concentration (one molecule of target DNA) in a defined sample however, there is concern over the detection of live versus dead cells because DNA may linger for prolonged periods after the death of the cell. A modification of the polymerase chain reaction has resulted in an efficient method for selective detection of live *Salmonell*a cells using quantitative PCR (qPCR) (Li and Chen, 2013). Many rapid identification

and confirmation methods of these techniques have been, validated, standardized and developed into commercial products by a number of manufacturers to be used in a simple and easy way (Lee *et al*., 2015).

These systems have contributed to improve accuracy, efficiency, and capacity in detecting *Salmonella spp.* and continue to play an important role in the food and industrial microbiology areas. However, while the obvious advantages of PCR assays are the rapid time, sensitivity and specificity of detection, there are many disadvantages as well. These include the need for expensive equipment and trained personnel, the use of extensive DNA clean-up chemistries before addition to the PCR reaction and the need to culture the samples to meet the limit of detection threshold (Bell *et al*., 2016).

The other rapid detection technique is the biosensor technology. Biosensors are detection/identification methods that do not require complicated and expensive assay steps. In this method a recognition signal is generated when a specific analyte binds to the biological recognition element. The signal can be a change in mass, oxygen consumption, potential difference, refractive index, pH, current, and other parameters (Lee *et al*, 2015). Various pathogen-detecting biosensors have been developed, among these, optical sensors, especially colorimetric sensors, allow easy-to-use, rapid (within 15 min), portable, and cost-effective diagnosis (Yoo and Lee, 2016). A review of some recent examples of optical biosensors and their advantages and limitations with future strategies to overcome the limitations can be found in Yoo and Lee (2016). It is anticipated that a biosensor technique may replace existing immunology and nucleic acid-based assays (Van Dorst *et al*., 2010; Yoo and Lee, 2016).

2.7.3 *Salmonella* identification using mass spectrometry

Identification of bacteria by mass spectrometry (MS) has been an active research area for decades (Anhalt and Fenselau, 1975). Matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is the most common technique used for bacterial analysis by MS. MALDI-TOF MS detects many different biomolecules, such as nucleic acids, peptides, proteins, sugars and small molecules. The identification of the isolated microorganisms using this technology is by generation of fingerprints of highly abundant proteins followed by correlation to reference spectra in a database. The sample for analysis by MALDI-TOF MS is prepared on MALDI target plate by mixing or coating with solution of an energy-absorbent, organic compound called the matrix (i. e. 3,5-dimethoxy-4-hydroxycinnamic acid) (Singhal *et al*., 2015). After drying the sample, within the matrix it is then ionized in an automated mode with a laser beam. Desorption and ionization with the laser beam generates singly protonated ions from analytes in the sample. The protonated ions are then accelerated at a fixed potential, where these separate from each other on the basis of their mass-to-charge ratio (*m/z*). The charged analytes are then detected and measured using time of flight (TOF) analyzers. A characteristic spectrum called peptide mass fingerprint (PMF) is generated for analytes in the sample. Identification of bacteria by MALDI-TOF MS is by either comparing the PMF of unknown bacteria with the PMFs contained in the database, or by matching the masses of biomarkers of unknown bacteria with the proteome database (Singhal *et al*., 2015).

MALDI-TOF MS has recently emerged as a powerful tool for the identification of clinical isolates but also applicable to identify food-associated bacteria specially food pathogens and complies with a variety of requirements for

food microbial laboratories (Pavlovic *et al*., 2013). It has been used for rapid screening and identification of important *Salmonella* enterica subsp. enterica serovars (Dieckmann *et al*., 2008). MALDI-TOF MS is the technique that has proved successful in identifying bacteria down to the species level and even identifying specific strains, also it is easy to operate and rapid as the analysis is as fast as 10 min from colony selection to identification. However, the reproducibility had been a major concern with this method, this can be due to sample preparation as the proteins extracted vary with the type of the matrix (solvent) used (Wang *et al*., 1998). This technique fails to identify mixtures of bacteria (Bell *et al*., 2016) therefore, a single colony or pure culture is generally required. However, as the naturally contaminated food typically contain a small number of *Salmonella*, therefore, the need for isolation of *Salmonella* from a high background flora is still required and challenging as several difficulties may be encountered during enrichment. For example, cells of *Salmonella* are outcompeted by the natural microbiota found in the food sample or *Salmonella* are outright inhibited by specialized metabolites, such as antibiotics, produced by these same organisms (Singer *et al*., 2009; Gorski, 2012). In addition, to that the limitation of the technology of MALD-TOF MS is that identification of new isolates is possible only if the spectral database contains peptide mass fingerprints of the type strains of specific genera/species/subspecies/strains (Singhal *et al*., 2015). Unfortunately, commercially available libraries currently lack the breadth and specificity that is ultimately needed to analyse complex matrices such as food.

An alternative to MALDI-TOF MS is separation and detection of bacterial proteins by high-performance liquid chromatography mass spectrometry (LC-MS). Recently, LC-MS of intact lysates has been used for *Salmonella* serovar-level identification (Bell *et al*., 2016). This method chromatographically separates the

intact bacterial proteins prior to detection by MS. Consequently, many more proteins and, by extension, more serovar-specific marker proteins are detected (McFarland *et al*., 2014). This demonstrates that serovar-level identification of *Salmonella* is possible by LC-MS however, it is slower than MALDI-TOF MS analysis (Bell *et al*., 2016).

The sensitivity and specificity of *Salmonella* detection methods in food samples largely depend on the background microflora, sample matrix, presence of non-culturable cells, and inhibitory substances (e.g. fats, proteins, polysaccharides and antibiotics) (Torrence and Isaacson, 2003; Lee *et al*., 2015). There is a great improvement in the sensitivity and the speed in which *Salmonella* is detected. However, there is still need for additional sample preparation and purification techniques for achieving advances in the specificity of the detection method.

2.7.4 *Salmonella* and the analytical vapour detection techniques

Bacteria are known to produce a range of volatile organic compounds (VOCs) (Schulz and Dickschat, 2007; Kai *et al*., 2009). The analysis of VOCs generated by bacteria has been used as a possible alternative method for the identification of pathogenic bacteria. However, it has been proven that the growth conditions include culture medium composition influence the detection of VOCs (Tait, 2013). Therefore, the natural generated VOCs during bacteria metabolites cannot be used as a marker for a specific pathogen as identification of bacteria *via* their VOC profiles would need specific experimental parameters (Tait, 2012; Tait *et al*., 2013).

Enzymatic assays have been used for many years in the characterization of viable microorganisms and used as a tool for detection, and identification purposes. In the early years, the common methods and techniques of detection

for the viable microorganism characterization relied on the reaction of bacterial enzymes to produce a visual or a spectrophotometric analysis. The bacterial enzyme cleaves the substrate to yield a colorimetric or fluorescent product or a product that results in change of pH. Ion mobility spectrometry (IMS) is a straightforward, analytical vapour detection technique. This technique has been used successfully for microbial VOC detections in headspace of microbial cultures to overcome the drawback on the methods that rely on measuring the colour change when an enzyme associated with the pathogen of interest reacts with a chromogenic substrate (Snyder *et al*., 1991a and b). The 2-nitrophenol was detected by IMS/MS in the head space of the sample due to the reaction between o-nitrophenyl-β-D-galactopyranoside (ONPG) and the in *vivo E. coli* β-galactosidase enzyme. Consequently, these findings gave preliminary support to the concept that vapour products that arise from reactions between the enzyme substrates and the bacterial enzymes could be detected and serve as markers for the presence of the specific pathogen. In addition, because many metabolites may be common to several microorganism species the incorporation of the synthesised enzymatic substrates containing the chosen metabolites into culture media has been proven to enhance the specificity of the identification of bacteria and enable differentiation of species (Orenga *et al*., 2009).

2.8 The analytical approaches for VOCs detection

The extensive literature concerning the use of VOC analysis for identification of pathogens is already the subject of various articles (Senecal *et al*., 2002; Panigrahi *et al*., 2008; Bhattacharjee *et al*., 2011; Guillemot *et al*., 2013; Tait *et al*., 2014 a and b,). The introduction of new analytical approaches and technological developments in instrumentation has enabled the detection of low concentrations of VOCs generated through hydrolysis of an enzymatic substrate.

For example, a gas chromatography–mass spectrometry (GC-MS) method that achieves nanomolar detection limits was reported (Han *et al*., 2008). Analysis of volatile compounds in foods is complicated due to the presence of highly complex mixtures of the VOCs. However, GC-MS has become the first choice for analysis of volatile compounds in food samples due to its high performance in the separation and identification of complicated and similar compounds (Cadwallader and MacLeod, 1998). The volatile analysis using this technique requires a prior sampling step, in which volatiles are isolated from the matrix and, if possible, pre-concentrated. Headspace solid phase microextraction (HS-SPME) is a popular method of sampling and pre-concentration of volatiles and semi-volatiles, which is being routinely used in combination with GC-MS (Soria *et al*., 2015). It is an inexpensive, solvent-free, and reliable technique with excellent sensitivity and good selectivity (Pinho *et al*., 2002). The main disadvantage of SPME is the limited number of commercially available stationary phases (fiber materials) (Merkle *et al*., 2015) however, there are available fiber types that cover the high scale of polarity of target analytes. The maximum sensitivity of SPME is at the equilibrium point, however, full equilibrium is not necessary to identify and quantify volatiles, because of the linear relationship between the amount of analyte absorbed by the SPME fiber and its initial concentration in the sample matrix under non-equilibrium conditions (Shang *et al*., 2002).

2.9 *Salmonella* metabolites and volatile organic compounds

Chemical analysis of bacterial metabolites has been introduced as bacterial differentiation and detection methods (Ehrhardt *et al*., 2010). Volatile organic compounds (VOCs) are produced as parts of microorganism's metabolic pathways. VOCs are a large and highly diverse group of carbon based molecules which are naturally volatile in ambient temperature with a minimum evaporate

pressure of 1 kPa (Dixon *et al*., 2011; Sohrabi *et al*., 2014). Bacteria produce a wide range of VOCs that can be characterized in a number of groups including fatty acids, aromatic compounds, nitrogen containing compounds and sulphur volatile compounds (Schulz and Dickschat, 2007; Tait, 2012). Recent advances in ionization technologies allow researchers to perform sensitive qualitative and quantitative analysis of high molecular weight compounds and low molecular weight compound analysis in biological experiments using GC/MS (Glish and Vachet, 2003). The analysis of VOCs generated by bacteria has been reported to be used as an alternative method for the identification of pathogenic bacteria (Tait *et al*., 2013; Tait *et al*., 2014 a, b). 2-Aminoacetophenone and indole are examples of usual VOCs that have been used as common markers for *Escherichia coli* and *Pseudomonas aeruginosa* (respectively) detection in culture media (Cox and Parker, 1979; Wang *et al*., 2001).

Headspace sampling using SPME technique followed by GC/MS analysis (HS-SPME GC/MS) has been widely applied to detect the key compounds and bacterial species implicated in food spoilage (Arnold and Senter, 1998). One example is the study performed for the qualitative analysis of volatile metabolites by *Salmonella typhimurium* on selective agar medium Trypticase Soy Yeast (TSY) at 35°C for 24 h. The identified *Salmonella* specific VOCs grown on TSY were, 3-methyl-1-butanol, dimethyl sulfide, 2-undecanol, 2-pentadecanol and 1-octanol (Senecal *et al*. 2002). An overview of recent research investigating the VOCs profile of *Salmonella* strains in different broths detected with GC/MS and other different analytical method and different VOC extraction method are shown in Table 2.1.

The evaluation of VOCs from bacterial pathogens has been enhanced and used to develop more sensitive and accurate methods to prove the absence or

presence of pathogens by application of VOC-labelled enzyme substrates that target specific enzyme activities of the bacteria under investigation; where the bacteria metabolise the substrate and liberate a specific VOC (Snyder *et al*. a and b; Lough *et al*., 2017). This concept of using enzyme substrates was extended by Strachan *et al*. (1995) and applied to detect bacteria in food samples, specifically *E. coli* using the substrate 2-nitrophenyl-β-D-glucuronide, *Aeromonas spp.* using the substrate 2-nitrophenyl-β-D-galactoside), *Listeria spp*. using the substrate 2-nitrophenyl-β-D-glucopyranoside and *Staphylococcus aureus* using the substrate 2-nitrophenyl-β-D-galactoside-6-phosphate each liberating the VOC 2-nitrophenol. Tait et at. (2014b) detected *L. monocytogenes* in milk samples using the commercially available 2-nitrophenyl-β-D-glucoside and the the synthesized 2-[(3-fluorophenyl) carbamoylamino] acetic acid, to liberate unique, identifiable and quantifiable 2-nitrophenol and 3-fluoroaniline through activity of β-glucosidase and hippuricase enzymes, respectively.

Enzyme substrates that are available to purchase generally incorporate compounds that are chromogenic or fluorogenic on release and are often unsuitable for VOC analysis. Therefore, there is a need to synthesis enzyme substrates, that are tagged with a compound that must be volatile enough to be detected in the headspace. Also, as many of the VOCs identified in bacterial culture are found to be common in pathogenic species of interest due to shared metabolic pathways therefore, it is important that the label VOC compound needs to be unnatural in its occurrence so can be accurately used as a marker to identify the presence or absence of the specific pathogen.

Table 2.1 Recent research investigating VOCs profile for different Salmonella strains in different broths detected with different analytical method and different VOC extraction method

***Salmonella* Strains**	**Culture medium**	**Sampling**	**Analytical method**	**VOCs detected**	**Reference**
S. typhimurium	Tryptic soya broth	Flushing the culture headspace for 1 min with CO_2 (99.99%; 2 litres/min) at room temperature	Secondary Electrospray Ionization-Mass Spectrometry (SESI-MS)	Acetic acid Acetone Acetonitrile Butanol Ethanol Ethylene glycol Indole Isopentanol 4-Methylphenol *2-Nonanone* 2-Pentanone Pyrimidin	Zhu *et al*., 2010
Salmonella enterica	Complex media structured by the DSMZ (www.dsmz.de) No 681	Developed dynamic headspace sampling system	Proton-Transfer-Reaction Mass Spectrometry (PTR-MS)	Acetaldehyde Acetic acid 1-butanol 2-butanone Ethanol Methanol Methanethiol 2-methyl-1-butanol	Bunge *et al*., 2008
S. typhimurium	Fresh alfalfa sprouts Luria Bertani broth consisting of 10 g Bacto tryptone incubated at 37 °C in a gyrotory shaker	HS-SPME a 75 µm (carboxen/PDMS) at 20 °C for15 min	GC/MS (SPB5, 30 m × 0.1 mm i.d., 0.25 µm) Helium (99.99%) 0.5 mL min−1 2 min at 40 °C and raised to 240 °C at a rate of 50 °C min−1 TOF range of 30–400 m/z, 70 eV	Dimethyl sulfide, Carbon disulfide, Ethyl acetate, Methyl alcohol, 2-Heptanol, 1-Propanol, 1-Pentanol and 1-Hepten-3-ol	Siripatrawan and Harte, 2007

cont'd table 2.1 Recent research investigating the profiles of *Salmonella* strains in different broths detected with different analytical method and different VOC extraction method

Salmonella Strains	Culture medium	Sampling	Analytical method	VOCs detected	Reference
S. arizonae, and S. gallinarum	(A cooked meat medium, Bacto-peptone -5.; Bacto-yeast extract.; D-glucose) incubated at 34 ºC for up to 10 weeks		Gas chromatography-nitrogen phosphorus selective detector (GC-NPD)	Dimethyl sulfide Dimethyl trisulfide Dimethyl tetrasulfide Ethanthioate Methyl sulfide	Jenkins *et al*., 2000
S. agama *S. arizonae IIIa* *S. arizonae IIIb* *S. brandenburg* *S. hader* *S. meleagridis* *S. enteritidis ATCC 13070 and* *S. typhimurium ATCC 7823*	Trypticase soy broth37 º C for 24 h One microliter of ethanol solution of 0.05% valeric acid ethyl ester and 3.7 g of NaCl were added	HS-SPME 100 µm PDMS (polydimethyl siloxane), 65 µm PDMS/DVB (divinylbenzene), and 75 µm PDMS/Carboxen fiber	GC-9A (DB-624) (30 m, 0.53 mm i.d., 3 µm film thickness Carrier gas was He The column temperature was held at 35 °C for 5 min, ramped to 200 °C at 3 °C/min, and then to 220 °C at 10 °C/min and held for 20 min	Only retention times were presented	Ogihara *et al*., 2000
Salmonella enteritidis (S.E)	Trypticase soy broth Plates incubated for 18 h at 35°C ($2.91x10^8$ cells mL^{-1})	HS-SPME (the PDMS fibre was allowed to equilibrate with the headspace volatiles for 30 min and then into the injector of the GC for 1 min desorption of entrapped VOCS	GC-MS (column 60 m x 0.25 cm(id), 0.25-µm film thicknessDB-1 And electronic nose	9-Decene-1-ol Dodecanol Ethanol 3-methyl -1-butanol Octanol 1-Propanol 1-tetradecanol Cis-7-Tetradecene-1-ol	Arnold and Senter, 1998

2.10 Salmonella enzymes

The *Salmonella* enzymes targeted in this study include α-galactosides (+), C-8 easterase (+), pyrrolidonyl peptidase (-) and decarboxylase.

2.10.1 Glycosidases

Glycosidase enzymes are enzymes that hydrolyse glycosides or break down a glycosidic bond to release the sugar part of the molecule, which is then used for generation of energy; the non-sugar part of the molecule is referred to as an aglycone. Detection of this free aglycone can be used to demonstrate the presence of a glycosidase which proves the presence of the specific organism. The nomenclature of the glycosidase enzyme is governed by the type of derivative it hydrolyses. For example, a galactosidase will hydrolyse derivatives of galactose. It is important to remember that these enzymes do not act on the sugar molecule itself but act on the glycosidic bond. This glycosidic linkage may have alpha or beta orientation and this will determine whether the enzyme is able to act or not. As a convenient assay for glycosidases, sugar may be linked to coloured dyes via the alpha or beta linkage to form chromogenic substrates that when hydrolysed release visible colour and this can demonstrate bacterial enzyme activity. Sugars may also be linked to fluorescent molecules to form fluorescent substrates. Hydrolysis of the substrates then leads to restoration of fluorescence which may be observed under a suitable ultra violet light source.

Salmonellae are Gram-negative bacteria that can ferment glucose belonging to the family Enterobacteriaceae. It has been long recognized (Perez *et al*., 2003) that *Salmonella* produce galactosidase enzymes, which only hydrolyse substrates containing galactose that is linked to another molecule or group via an alpha linkage and not a beta linkage (Perry *et al*., 1999; Perry and

Ford, 2002). One of the best known beta galactosides is *o*-nitrophenyl-β-D-galactopyranoside (ONPG), which comprises galactose in the beta linkage with a yellow compound, *o*-nitrophenol. Hydrolysis leads to generation of a bright yellow colour. The ONPG test is a very useful test for differentiating *Salmonella* (-) from many other genera within Enterobacteriaceae (Smith *et al*., 1972). Another galactoside that is linked via the alpha linkage is phenyl α-D-galactopyranoside which is hydrolysed by alpha galactosidase; the hydrolysis leads to the release of galactose and an aglycone known as phenol. Phenol is a volatile compound that can be detected on headspace of a sample using HS-SPME GC/MS and this is the substrate used in this project.

2.10.2 Esterases

It has been established that *Salmonella* strains possess esterase activity, and hydrolyse long chain esters specially (C-8) and (C-9) derivatives; this activity is an excellent diagnostic marker for the discrimination of *Salmonella* (+) from most other bacteria (-) (Aguirre *et al*., 1990). Therefore, recently a number of media have been manufactured which rely on the detection of esterase activity by *Salmonella* using chromogenic and fluorogenic substrates (Cooke *et al*., 1999, Eigner *et al*., 2001; Freydiere and Gille, 1991). In chromogenic and fluorogenic substrates removal of the carboxylic acid by hydrolysis of the ester link is accomplished by bacteria esterases and results in generation of colour or fluorescence, respectively. On this basis C-8 esterase substrates are designed to use for the identification of *Salmonella* by detecting a volatile compound released during their C-8 esterase activity using HS-SPME GC/MS. As a convenient assay for good identification of *Salmonella* using C-8 esterase activity and GC/MS technique, the volatile compound part in esters may be labelled with

a halogen atom, such as, chlorine or fluorine atoms. The C-8 esterase substrates used in this project were either purchased or synthesised (Chapter 3, Section 3.11).

2.10.3 Peptidases

Peptidases involved in the breakdown of the peptide linkage exist between amino acids. Chromogenic peptidase substrates have been devised that link an amino acid to a coloured dye to form a colourless enzyme substrate (James *et al*., 2007). Hydrolysis of this substrate then releases the amino acid, which is used for metabolism, and the coloured dye which is used to identify the presence of specific organism. There are many types of peptidase substrates that can be useful for detection and identification of organisms in different samples. For example, aminopeptidase substrates generated by linking the amino group of p-nitroaniline to the carboxyl group of one of other amino acids are the most widely used chromogenic substrates. The enzyme responsible for the hydrolysis is referred to as an aminopeptidase (Bennett *et al*., 1999). As well as chromogenic substrates for the demonstration of aminopeptidase activity, fluorogenic substrates based on amino acids linked to 7-amino-4-methylcoumarin are commercially available and widely used (Maly *et al*., 2002; Kato *et al*., 1978).

In the same manner L-pyroglutamic acid can be linked to p-nitroanaline to form a chromogenic substrate for detection of pyroglutamyl aminopeptidase also known, as pyrrolidonyl peptidase (PYRase). Detection of PYRase is highly useful for differentiation of *Salmonella* from many other genera of Enterobacteriacese. *Salmonella* have no enzyme pyrrolidonyl peptidase (PYRase) activity and are well known to be PYRase-negative (Bennett *et al*., 1999; Ford, 2010). This negative activity has been reported as a distinctive test for *Salmonella* from other

bacteria in food samples (Bennett *et al*., 1999; Wenke, 2009). In this project the PYRase activity has been adapted for better selective detection of *Salmonella*. The synthesized PYRase substrate (L-pyrrollidonyl fluoroanilide (Chapter 3, Section 3.11) was used in this project.

2.10.4 Decarboxylases

A useful additional screen for *Salmonella* is the use of lysine and ornithine in growth media where these two amino acids will be decarboxylated by *Salmonella*. The enzyme ornithine decarboxylase catalyses the decarboxylation of ornithine to form putrescine or tetramethylenediamine ($NH_2(CH_2)_4NH_2$). While the enzyme lysine decarboxylase catalyses the decarboxylation of lysine to form cadaverine or pentamethylenediamine ($NH_2(CH_2)_5NH_2$) and liberate carbon dioxide.

Salmonella will use the amino acids lysine and ornithine as a source of carbon and energy for growth. If lysine and ornithine are used, *Salmonella* will accumulate alkaline/basic metabolic products (putrescine and cadaverine) (Tan and Shelef, 1999). The enzyme ornithine and lysine decarboxylases degrade lysine and ornithine to produce these alkaline/basic products however, the enzymes do not do this unless the growth medium is acidified by other metabolic activities (Gale and Epps, 1944). This is can be done by adding glucose to the growth media where *Salmonella* will acidify the medium by using the glucose present to cause the pH to drop due to the rapid production of pyruvic acid (Bowden *et al*., 2009); then the lysine and ornithine decarboxylase enzymes can metabolize the lysine and the ornithine. Cadaverine and putrescine are also important factors in food quality because their presence in food, especially in fish, cheese and meat products are determined by the food-processing and microbial

factors involved. Also cadaverine and putrescine are important factors in food poisoning as they amplify the toxic effects of histamine (Armağan, 2007). Because their production increases when the bacterial population increases, these amines (together with histamine) have been used as indicators of fish quality (Ryser *et al*., 1984). So, generally the levels of concentration of biogenic amines give an indication of the levels of microbiological concentration in food products and hence can be used as a reliable quality indicator (Shalaby, 1996).

In GC analysis, compounds containing functional groups with active hydrogens such as -NH are of primary concern, because of the tendency of these functional groups to form intermolecular hydrogen bonds (Zaikin and Halket, 2003). These intermolecular hydrogen bonds affect the inherent volatility of compounds containing them, their tendency to interact with column packing materials and their thermal stability (Sobolevsky *et al*., 2003). So putrescine and cadaverine released during *Salmonella* activity need to be derivatized as part of their sample preparation for gas chromatography (GC) analysis to obtain an accurate and reliable chromatographic result in terms of separation and detection. Derivatization by acylation is a popular reaction for production of volatile derivatives and also acyl derivatives tend to produce fragmentation patterns of compounds in MS applications which are clear to interpret and provide useful information on the structure of these materials (Orata, 2012). Different derivatizing reagents have been used for spectrophotometric, spectrofluorimetric and electrochemical detection (Cichy *et al*., 1993; Khuhawar and Rajper, 2003; Schenkel *et al*., 1995). However, a few gas chromatographic (GC) methods have been reported for the determination of putrescine and cadaverine and some of them are summarised in Table 2.2.

Table 2.2 Quantitaive and qualitative analysis of biogenic amines

Matrix	technique	Method/ procedure	pH	derivatizing reagent	Reference
Standards putrescine and cadaverine and Burgundy wine	SPME-ED-GC thermionic specific detection (TSD)	The three electrode system	Borate buffer pH 8	No derivatization	Conte and Miller, 1996
Standards putrescine and cadaverine in DCM	GC (the split liner treated with KOH solution)	Cold on-column injection	N/A	Direct GC analysis without derivatization	Bonilla *et al*., 1997
Serum of cancer patients	GC Optimum	Solvent extraction	pH range 3–10 optimum at pH 6.75	Trifluoroacetylacetone	Khuhawar *et al*., 1999
Histamine in fish	GC	Extraction with alkaline methanol	NaOH 0.1 N pH 9 or 10	Direct GC analysis	Hwang *et al*., 2003
Standards putrescine and cadaverine in organic solvents	SPME GC/MS	On-fibre and liquid-phase derivatisation	N/A	Trifluoroacetylacetone	Awan *et al*., 2008
Standards putrescine and cadaverine in organic solvents	GC-MS	Aqueous and non-aqueous phase derivatisation	Phosphate buffer pH 7	Trifluoroacetylacetone	Awan, 2008

The GC methods involve mostly isobutyloxycarbonyl, pentafluoropropionyl, trifluoroacetyl and heptafluorobutionyl derivatives (Jiang, 1990; Teti *et al*., 2002; Khuhawar *et al*., 1999). The trifluoroacetylacetone (TFAA) is the derivative reagent chosen to be used because it is easily available and the presence of the trifluoromethyl group is reported to enhance the volatility of derivatized molecules (Uden, 1984; Khuhawar *et al*., 1999). The reaction of TFAA and putrescine and cadaverine is governed by the pH of the solution. As the pKa values for the amino groups in putrescine are 9.35 (+2) and 10.92 (+1) and for cadaverine are 10.05 (+2) and 10.92 (+1) in aqueous solution (broth) (Dean, 1985) therefore, in the broth these compounds exist as protonated diamines. The protonated diamines form are needed to convert to the free base form to react with the derivative reagent (TFAA). Therefore, the pH of solution during the reaction needs to be raised to 10 or above.

2.11 Summary

In brief, this chapter contains information about *Salmonella* and its detection/identification methods. Introduction to *Salmonella* as a very important and widespread pathogen and a major cause of concern mainly for the food industry as it causes the largest number of outbreaks was reviewed. The food associated with this pathogen was discussed with the most common serotypes associated with human illness. The inspection of food for the presence of *Salmonella* has become routine all over the world. The methods have been developed for many foods having prior history of *Salmonella* contamination was discussed. Traditional methods for isolating and identifying *Salmonella* in foods rely on pre-enrichment, selective enrichment in selective and differential media, biochemical tests, and serological confirmation. The more rapid *Salmonella* detection methods developed, which differ mainly in technique was discussed.

Due to the low infective dose of *Salmonella,* methods for its detection are required to prove the presence of one cell in a defined food sample. Many new methods are constantly being rapid and sensitive however, lack specificity. Investigation to other methods that generating specific VOC biomarkers which have the potential to be more robust and specific for *Salmonella* has been provided. Specifical focus on the use of HS-SPME-GC-MS technique and use of enzyme substrates that will liberate exogenous VOC biomarkers for the detection of pathogenic *Salmonella* are made. The key enzymes that produced by *Salmonella* which could prevent detection of false positive results and encouraging the applicability of the assay was also discussed.

Chapter 3: Experimental

3.1 Introduction

Different detection method of foodborne pathogen *Salmonella* were reviewed in the last chapter. The analysis of the bacterial VOCs in the headspace of samples using solid phase microextraction (SPME) gas chromatography coupled to mass spectrometry (SPME-GC-MS) is the technique used lately with advantages, and was applied in this study. This chapter includes a description of the experimental procedures carried out in order to reach the objectives of this thesis. All the chemicals, bacteria strains and the bacteria media used in the experiments conducted to develop an analytical detection method that applied to detect *Salmonella* in food samples are described in this chapter. Headspace sampling method for the generated VOCs is described below. The analytical techniques used for screening the VOCs and identifying the isolated organisms are given in this chapter. Evaluation methods and food analysis process are provided. The synthesize procedures of some enzymes substrates used are given below.

3.2 Chemicals and reagents

1-Decanol (99%; CAS No. 112-30-1), 1-dodecanol (98%; CAS No. 112-53-8), ethyl decanoate (99%; CAS No 110-38-3), ethyl octanoate (99%; CAS No 106-32-1), 2-heptanone (99%; CAS No. 110-43-0), 2-phenylethanol (99%; CAS No. 60-12-8), 1-tetradecanol (97%; CAS No. 112-72-1), 2-tridecanone (99%; CAS No. 593-08-8), 3-methyl-1-butanol (98%; CAS No. 123-51-3), 9-decen-1-ol (97%; CAS 3019-22-2), para-tolyl-octanoate (CAS No. 59558-23-5), isobutyl octanoate (MDL No. CDS000565), 1-hexanol (98%; CAS No.111-27-3), 3-fluoroaniline (99%; CAS No. 372-19-0), N-methyl-2-pyrrolidinone (99%; CAS No. 872-55-1), L-ornithine monohydrochloride (99%; CAS No.3184-13-2),

triethylenamine (99%; CAS No. 121-44-8), phenol (99%; CAS No. 108-95-2), isobutanol (2 methyl-1 propanol) (99.8%; CAS No. 78-83-1), L-lysine hydrochloride solution (100 mM amino acid in 0.1 M HCl; CAS No. 10096-89-2), dichloromethane (DCM) (99.8%; CAS No. 75-09-2), triethylenamine (99%; CAS No. 121-44-8), 2,6-dimethylphenol (99.5%; CAS No. 576-26-1), dimethylformamide (DMF) (99.8%; CAS No. 68-12-2), L-pyroglutamic acid (99.0%; CAS No. 98-79-3), 2-chloro-4-methylphenol (97%; CAS No. 6640-27-3), 2-chlorophenol (99%; CAS No. 95-57-8), 2-methylphenol (99.9%, CAS No. 95-57-86), 2-nitrophenol (98%; CAS No. 88-75-5). Cadaverine (1, 5-diaminopentane) (96.5%; CAS No. 462-94-2), potassium ferricyanide(III) (99%; CAS No. 13746-66-2) and putrescine (1,4-Butanediamine) (98.5%; CAS No. 110-60-1), sodium 1,2-naphthoquinone-4-sulfonate (97% CAS No. 521-24-4), 4-aminoantipyrine (99%; CAS No. 83-07-8), 4-Methylumbelliferone (89 %, CAS No. 90-33-5), vancomycin (CAS No. 1404-93-9), sodium chloride (99%; CAS No; 7647-14-5), sodium hydroxide (79 % ; CAS No; 1310-73-2), and agarose (CAS No; 9012-36-6) were obtained from Sigma Aldrich Ltd. (Gillingham, UK) while 1-octanol (99.7%; CAS No. 111-87-5), and novobiocin sodium salt (93 %; CAS No. 1476-53-5), were obtained from Fluka Ltd. (Gillingham, UK).

6-Chlorohexanol (97%; CAS No. 2009-83-8), octanoyl chloride (99%; CAS No.111-64-8), 2,2,2-trifluoroethanol (99%; CAS No. 75-89-8), p-cresol (4-methylphenol) (99%; CAS No. 106-44-5), lithium chloride anhydrous (99%; CAS No. 7447-41-9) and isobutyl chloroformate (IBCF) (89.0%; CAS No. 543-27-1) were obtained from Alfa Aesar (Heysham, UK). While N-methylmorpholine (99.0%; CAS No. 109-02-4) was purchased from Lancaster Synthesis (Middlesex, UK). Hexyl octanoate (97%; CAS No. 1117-55-1) was purchased from SAFC Sigma Aldrich (Steinheim, Germany). Phenyl α-D-galactopyranoside (CAS No.

2871-15-0), 4-Methylumbelliferyl caprylate (CAS No. 20671-66-3) were purchased from Glycosynth (Warrington, UK). Tris hydroxymethyl aminoethanol (99.9%; CAS No 77-86-1) was obtained from Melford (Suffolk, UK). Erythromycin (93%; CAS No. 117-07-8) was obtained from Duchefa biochemie b.v (Haarlem, The Netherlands). Phenoxymethyl octanoate (GT378) was made by a PhD student in our Laboratory. All chemicals and reagents were stored and kept as directed and labeled.

3.3 Bacteria media

Brain heart infusion (BHI) broth (CM1135) was obtained from Oxoid Ltd. (Basingstoke, UK). The broth is composed of BHI solids (12.5 g), beef heart infusion solids (5.0 g), proteose peptone (10.0 g*),* glucose (2.0 g), sodium chloride (5 g), and di-sodium phosphate (2.5 g). The pH of this broth is 7.4 ± 0.2 at 25 ◦C. Tryptone soya (TS) broth (CM0129) was also obtained from Oxoid Ltd. (Basingstoke, UK). This broth is composed of pancreatic digest of casein (17.0 g), an enzymatic digest of soya bean (3.0 g), glucose (2.5 g); the pH of the broth at 25 °C is 7.3 ± 0.2. Tryptone soya agar (TSA) (CM0131) was also obtained from Oxoid Ltd. (Basingstoke, UK). This broth is composed of pancreatic digest of casein (15.0 g), enzymatic digest of soya bean (5.0 g), and sodium chloride (5.0 g), agar (15 g); its pH is 7.3 ± 0.2 at 25 °C.

Rappaport-Vassiliadis Soya Peptone (RVS) Broth (CM0866) was obtained from Oxoid Ltd. (Basingstoke, UK). RVS broth is a selective enrichment medium for the isolation of *Salmonellae* from food. The typical formula of the broth is soya peptone (4.5 g), sodium chloride (7.2 g), potassium hydrogen phosphate (1.26 g), di-potassium hydrogen phosphate (0.18 g), magnesium chloride (anhydrous) (13.58 g) and malachite green (0.036 g). The pH of this broth is 5.2 ± 0.2 at 25◦C. Buffered Peptone Water (BPW) (CM0509) was also obtained from Oxoid

(Basingstoke, UK). The typical formula of BPW per litre is peptone (10.0 g), sodium chloride (5 g), disodium phosphate (3.5 g) and potassium dihydrogen phosphate (1.5 g). The pH of BPW is 7.2 ± 0.2 at 25°C. Agar plates of Harlequin™ *Salmonella* ABC Medium (HAL001) obtained from Lab M Limited (Lancashire, UK), and agar plates of CLED medium (CM0301) obtained from Oxoid Limited (Basingstoke, UK) were kindly provided by Prof John Perry, Freeman Hospital (Newcastle upon Tyne, UK). The formula of *Salmonella* of ABC medium per litre is beef extract (5.0 g), peptone (5.0 g), sodium citrate (8.5 g), sodium desoxycholate (5.0), agar (12.9 g), substrate of 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside (X-α-Gal) 0.08 g), substrate of 3,4-cyclohexenoesculetin-b-D-galactoside (CHE-ß-Gal) (0.3 g), Ferric ammonium citrate (0.5 g), IPTG (0.03 g). The typical formula of CLED medium per litre is peptone (4.0 g), `Lab-Lemco' powder (3.0 g), tryptone (4.0 g), lactose (10.0 g), L-cystine (0.128 g), Bromothymol blue (0.02 g), and agar (15.0 g). The pH of CLED medium is 7.3 ± 0.2 at 25 °C.

3.4 Bacteria strains

The research was done on bacteria that are hazard group 2 organisms. Bacteria were kindly provided by Professor John D. Perry at the Microbiology Department, Freeman Hospital, Newcastle upon Tyne. Six strains of the Gram negative bacteria *Salmonella* were provided in glycerol stocks; they were: *Salmonella enterica* serovar London (*S. London*), *Salmonella enterica serotype Oranienburg (S. Oranienburg), Salmonella Typhimurium* (*S. Typhimurium*), *Salmonella Stanley (S. Stanley)*, *Salmonella* serovar *Gallinarum* (*S. Gallinarum*), Salmonella *Othmarschen* (*S. Othmarschen*). Gram-positive bacteria *Listeria monocytogenes* (NCTC 11994) and *Listeria monocytogenes* (NCTC 105376) and other Gram-negative-bacteria; *E. coli* (NCTC 10418), *E. coli* (K -12), *E. coli*

(NCTC 18039), *E. coli* (NCTC 10213), *E. coli* (O157: H), *Pseudomonas aeruginosa* (DSMZ 19980), *Pseudomonas aeruginosa* (NCTC 10662*), Campylobacter Jejuni* (NCTC 11322) were provided on agar plates. Gram negative bacteria include *Enterobacter cloacae* (NCTC 11936), *Cronobacter sakazakii* (ATCC 29544), *Klebsiella oxytoca* (Wild strain) and *Serratia marcescens* (NCTC 10211), and Gram positive bacteria include *Enterococcus faecalis* (NCTC 775), *Enterococcus faecium* NCTC 7171, *Streptococcus salivarius* (NCTC 8618) were provide on blood agar plates to test their enzyme activities as representatives of the species isolated from food samples.

3.5 Food samples

Food types analyzed here were raw chicken meat, eggs, cheese and milk. Four samples of fresh raw chicken meat; two are British Oakham chicken skinless and skin-on breast fillets from Marks and Spencer (Newcastle, UK), another two are chicken wings and free range (British chicken thighs & drumsticks thighs) are from ASDA (Gosforth, UK). Four milk samples were collected and analysed, two samples (whole milk and semi skimmed milk were collected from Marks and Spencer (Newcastle, UK). Another two are goat`s milk and jersey full cream milk were collected from ASDA store (Gosforth, UK).

Three eggs samples, free range, organic and caged hen eggs were collected from ASDA store (Gosforth, UK). Six cheese samples two of them were made from unpasteurized milk; Brie de meaux cheese and Roquefort AOP cheese and collected from Fenwick (Newcastle, UK). Goat's milk cheese and cheddar cheese were collected from ASDA store (Gosforth, UK). Handmade Colston Bassett Stilton cheese and Claxton Blue cheese were obtained from Marks and Spencer (Newcastle, UK).

3.6 Bacterial growth and sample preparation

3.6.1 Preparation of agar plates bacteria

Peptone soya agar (TSA) was the nutrient agar used for supporting the growth of bacteria. TSA was prepared as directed by manufacturer and sterilized using Ambassador Autoclave at 120 °C for 15 min. After removal from the autoclave, agar media was cooled to 50 °C in a water bath and dispensed as soon as possible on plates to avoid prolonged exposure to heat which result in autoxidation and the formation of superoxides. The prepared plates were then stored in a fridge (2-8 °C) and used as needed.

Bacterial stocks were streaked onto nutrient agar plates (TSA) and the plates were incubated overnight at 37 °C to allow for the cultures to grow. Culture dishes were wrapped with laboratory plastic sealing film and stored upside down (agar side up) to minimize contamination and to keep both the culture and agar properly hydrated in a fridge at 2-8 °C for daily and weekly use.

3.6.2 Preparation of bacteria suspension and bacterial samples

Bacteria strains were sub-cultured on peptone soya agar plates one day prior to preparation for VOC analysis. After overnight incubation at 37 °C a single colony was harvested using a sterile loop (the loop was flame sterilized for 30 s using bunsen burner) and transferred to a 20 mL clear vial with PTFE septum and screw cap contain 10 mL of sterile nutrient broth. All nutrient broths (BHI, TSB, and RVS) were made up according to manufacturer`s guidelines and appropriate volumes (10 mL) were dispensed into 20 mL clear vials with PTFE septum and screw cap. The vials were then sterilised using Ambassador Autoclave at 120 °C for 15 minutes and stored in the fridge (2-8 °C) and used as needed. The inoculated vial was incubated at 37 °C for a while in order to prepare the bacterial suspension to use in preparation of bacterial samples.

Bacterial samples were prepared by measuring the absorbance of the incubated bacterial suspension at OD_{600nm} at an absorbance reading of 0.132 (equivalent to 0.5 McFarland units (CFUs) / mL broth). An aliquot of 100 µLof bacterial suspension (1.5×10^8 CFUs) was added to a 20 mL clear vial with PTFE septum and screw cap containing 10 mL sterile broth. Inoculated broth samples were then incubated straightforward for 18-24 hours at 37 °C and subjected to volatile profiling via HS-SPME-GC–MS. A blank sterile broth was also sampled via the HS-SPME method after incubation at 37 °C for 18 h. The preparation of all bacterial samples followed this procedure.

3.6.3 Preparation of successive serial dilutions

The first step in making a serial dilution was started with making a10 fold dilution and then the process was repeated to make successive serial dilutions as follows: A known volume (100 µL) of stock bacterial culture ($1X10^8$ CFU/mL) was prepared (as described in Section 3.6.2) and placed into a known volume (0.9 mL) of sterile saline solution 0.85%. This produced 1 mL of the dilute solution (1×10^7 CFU/mL). This dilute solution has 100 µL of extract / 1 mL, producing a 10-fold dilution. This single dilution is repeated sequentially using more and more dilute solutions as the "stock" solution. At each step, 100 µL of the previous dilution is added to 0.9 mL of sterile saline solution 0.85%. Each step results in a further 10-fold change in the concentration from the previous concentration. The bacterial samples were prepared by transfer 100 µLof a proper volume (usually 100 µL) of a proper diluted solution into (10 mL) final volume to produce the desired and appropriate concentration. Figure 3.1 illustrates these preparation steps.

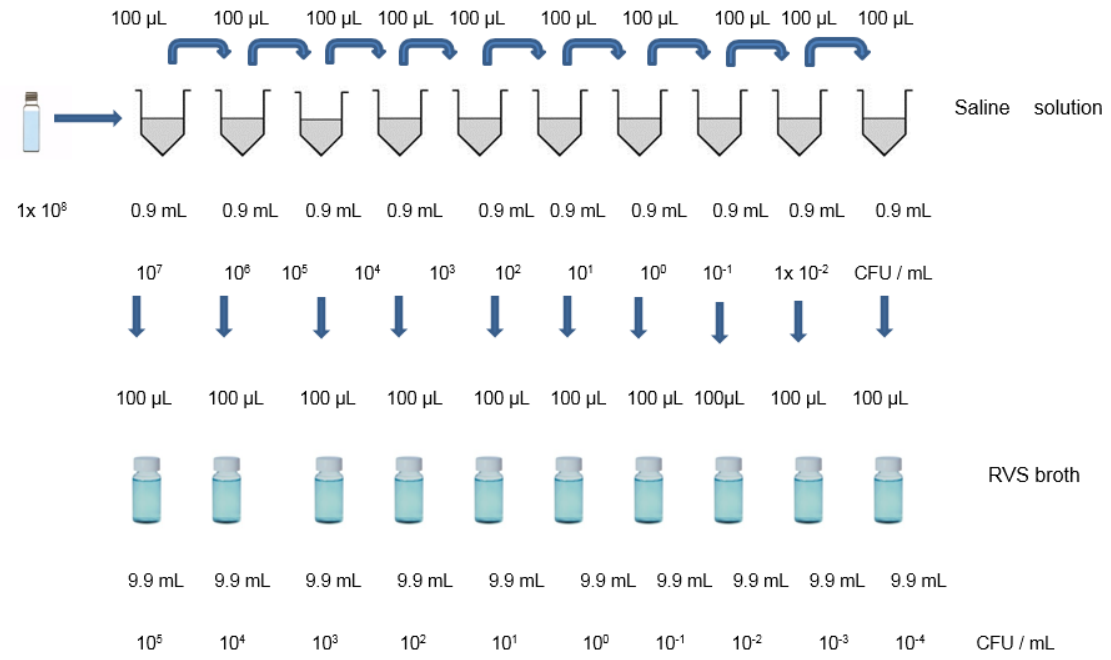


Figure 3.1 Successive serial dilutions scheme

3.6.4 Plate count method

The peptone soya agar plates were prepared as in Section 3.6.1 and inoculated with, 100 µL of examined level of bacteria for example; 1 x 10^0 and 1 x 10^1 CFUs / mL of S. stanley and dispersed in 2 drops around the center of the plates and with an around movement the inoculum spread evenly around the plates. The plates did not invert until all the liquid has been absorbed into the surface of the agar. The plates were incubated for a desired time 5 hours, 10 hours, and 18-24 hours at 37 ºCelsius. The plate count was performed after each certain incubation time by counting the number of colonies in each plate and average the number. To determine the presence and the level of viable organisms were in the original sample by multiply the average of the number of colonies by 10 because only 0.1 mL of 1 mL sample was plated.

3.7 Sampling

Sampling was performed using headspace solid phase microextraction (HS-SPME). SPME fibers evaluated for extracting bacterial VOCs were 100 µm polydimethylsiloxane (PDMS) and 85 µm polyacrylate (PA) (Supelco, Bellefonte, PA). The fibers were conditioned in the GC injection port before use, as directed by manufacturers' guidelines, and were used with a manual holder.

After the inoculated broths were incubated for 18-24 hours at 37 °C they were then placed in a 37°C water-bath for 10 min before sampling for headspace VOC equilibration. The PDMS/PA fiber was inserted through the septum of the sample vial`s caps and allowed to equilibrate with the headspace volatiles for 10 min. The fiber was then retracted into the barrel of the syringe and immediately inserted into the injection port of the GC for 2 min desorption of the entrapped VOCs. All experiments were conducted in triplicate.

3.8 Instrumentation

3.8.1 GC–MS analysis

Analysis of bacterial VOCs was achieved by Gas chromatography / mass spectrometry (GC/MS) using electron impact ionization. GC/MS analysis was performed on a Thermo Finnegan Trace GC Ultra and Polaris Q ion trap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) fitted with a polar GC column (VF-WAXms 30 m x 0.25 mm x 0.25 µm) or a non-polar GC column (HP-5MS 30 m x 0.25 x 0.25 µm) (Hewlett Packard, UK). The GC-MS system was operated with Xcaliber 1.4 SRI software.

Separation of bacterial VOCs on both GC columns was achieved using the following temperature program: initial 50 °C with 2 minutes hold ramped to 220 °C at 10 °C/min and then held for 10 minutes. The split-splitless injection

port was held at 230 °C for desorption of volatiles in split mode at a split ratio of 1:10. Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min**.**

The MS parameters were as follows: full-scan mode with scan range 50-650 amu at a rate of 0.58 scans / s. The ion source (electron-ionization (EI) mode) temperature was 250 °C with an ionizing energy of 70 eV and a mass transfer line of 250 °C.

Identification of VOCs was achieved using the National Institute of Standards and Technology (NIST) reference library (NIST Mass spectral library, version 2.0a, 2001) as well as the comparison of the retention time (t_R) and mass spectra of authentic standards. In addition to the mass spectral library that was built by a colleague.

3.8.2 MALDI–TOF MS analysis

Bacteria colonies were identified to species level using Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI–TOF MS). The instrument used was the Bruker Biotyper (Bruker, Coventry, UK).

The acquisition and analysis of mass spectra was performed by a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0) with the reference database version 3.1.2.0 (3,995 database entries; Bruker Daltonik) and default parameter settings (positive linear mode; laser frequency, 60 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 16.7 kV; lens voltage, 7.0 kV; mass range, 2,000 to 20,000 Da). For each spectrum, 240 laser shots in 40-shot steps from different positions of the sample spot were accumulated and analyzed (automatic mode, default settings). The Bruker bacterial test standard (Bruker Daltonik) was used for daily calibration according to the instructions of the manufacturer. MALDI-TOF MS data

interpretation were by using the Biotyper software which compares each sample mass spectrum to the reference mass spectra in the database, calculates an arbitrary unit score value between 0 and 3 reflecting the similarity between sample and reference spectrum, and displays the top 10 matching database records. As specified by the manufacturer, identification scores of ≥2.0 were accepted for a reliable identification to the species level (green), and scores of ≥1.7 but <2.0 were accepted for identification to the genus level (yellow). Scores below 1.7 were considered unreliable (red). This analysis was provided by Professor John Perry at the Microbiology Department, Freeman Hospital, Newcastle upon Tyne.

3.9 Analysis

3.9.1 Analysis of Salmonella VOCs

After *Salmonella* samples were prepared, as described in Section 3.6.2, inoculated vials were incubated for 18-24 hours at 37 °C and then subjected to volatile profiling via HS-SPME-GC-MS. For quantitative analysis of bacterial VOCs, in *Salmonella* strains, calibration graphs for all VOCs detected were prepared by spiking standards of a known concentration into 10 mL of blank culture media, followed by incubation at 37 °C in a water bath for 10 min and subsequent extraction of VOCs from the headspace. The HS-SPME procedure and GC–MS parameters for *Salmonella* samples and standards were consistent through all the analyses. The calibration curves were constructed with concentrations and peak area responses for quantitative determination of VOCs liberated by *Salmonella* strains. VOCs were quantified by using external calibration (Analysis, 2010) and the values for the limit of detection (LOD) and limit of quantitation (LOQ) were determined as the peak area 3 times the signal-to-noise ratio and 10 times the signal-to-noise ratio, respectively.

3.9.2 Evaluation of enzyme activities

A fresh stock solution of each substrate tested was prepared in an appropriate solvent. The stability of the substrate and the activity of the bacteria on each substrate were tested at an optimum concentration of 100 µg / mL. The bacteria samples were prepared, as described in Section 3.6.2, and the appropriate volume of tested substrate was added to the bacterial samples before incubation for 18-24 hours at 37 °C. The HS-SPME sampling and GC/MS analysis were performed as described in Sections 3.7 and 3.8.1, respectively. For a method control a blank of sterile broth (RVS, BHI or TSB) contained the same concentration of the substrate that applied to the bacteria samples was prepared, sampled and analyzed in a similar manner as the bacterial samples.

3.9.2.1 Evaluation of α-galactosidase activity

A stock solution of phenyl α-D-galactopyranoside (Figure 3.1) was prepared in deionized water at a concentration of 100,000 µg / mL each day of use. The activity of α-galactosidase for all *Salmonella* strains was tested using 100 µg /mL of this substrate. In addition, some other organism studied against *Salmonella* were tested using the same substrate.

3.9.2.2 Evaluation of pyrrolidonyl peptidase (PYRase) activity

A stock solution of L-pyrrollidonyl fluoroanilide (Figure 3.1) was prepared in N-Methyl-2-pyrrolidone (NMP) at concentration of 100,000 µg/mL each day of use and tested on bacteria strains using 100 µg/mL of the substrate. This substrate was tested on *Pseudomonas aeruginosa* to illustrate the reliability of the method as *Salmonella* strains are PYRase-negative.

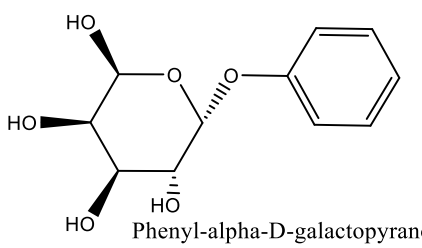
3.9.2.3 Evaluation of stability and activity of C-8 esterase substrates

A group of commercial and synthesized C-8 esterase substrates were tested for their stabilities in broths and activities with *Salmonella* strains. The C-8 esterase substrates stock solutions were prepared by dissolving an appropriate weight in an appropriate volume of NMP. The samples were prepared, and analyzed as described in Section 3.9.2. These substrates include commercial p-tolyl octanoate known as p-cresyl octanoate or p-methyl phenyl octanoate (Figure 3.2), commercial isobutyl octanoate (Figure 3.2), and commercial hexyl octanoate (Figure 3.2). Synthesized phenoxy methyl octanoate (Figure 3.2), synthesized 2,2,2-Trifluoroethyl octanoate (Figure 3.2), and synthesized chlorohexyl octanoate (Figure 3.2) were tested for their stability on broth and esterase activities on *Salmonella* strains.

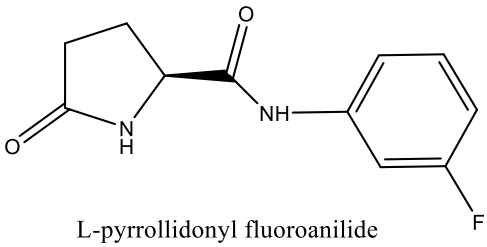
More C-8 esterase substrates that could release exogenous VOCs were synthesized and tested on *Salmonella strains*. These substrates include the following phenolic substrates: 2,6 dimethyl phenyl octanoate, 2-methyl phenyl octanoate, 2-chloro-4-methylphenyl octanoate, 2-nitrophenyl octanoate and 2-chlorophenyl octanoate. The structures of all these substrates were as shown in Figure 3.3.

3.9.2.4 Evaluation of decarboxylases activity of *Salmonell*a strains

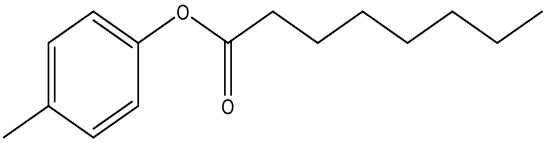
The enzyme ornithine decarboxylase present in *Salmonella* catalyses the decarboxylation of ornithine to form putrescine while the enzyme lysine decarboxylase present in *Salmonella* catalyses the decarboxylation of lysine to form cadaverine (Tan and Shelef 1999) and liberate carbon dioxide as can be seen in Scheme 3.1. A stock solution of L-ornithine monohydrochloride and L-lysine hydrochloride were prepared in deionized water at a concentration of 100000 µg / mL each day of use. And a 100 µg/mL of this solution was used to test *Salmonella* samples.



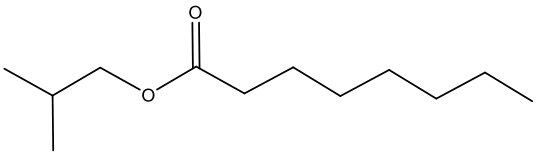
Phenyl-alpha-D-galactopyranoside



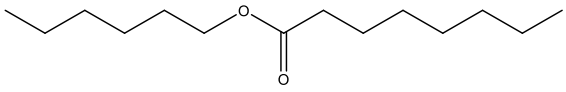
L-pyrrollidonyl fluoroanilide



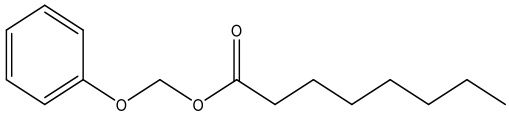
p-Tolyl octanoate



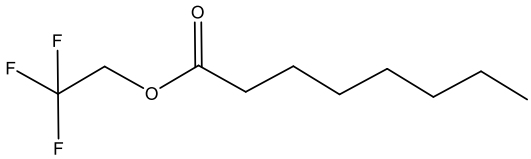
isobutyl octanoate



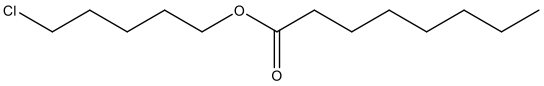
hexyl octanoate



phenoxymethyl octanoate

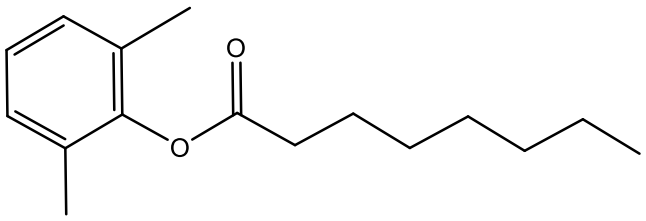


2,2,2-Trifluoroethyl octanoate

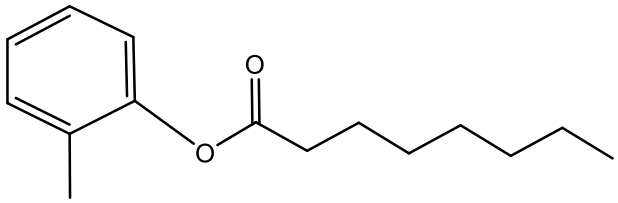


chlorohexyl octanoate

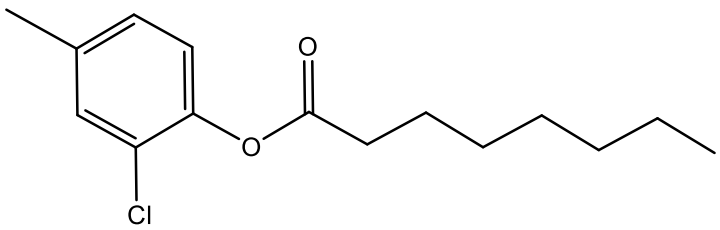
Figure 3.2 Structures of some evaluated enzymatic substrates



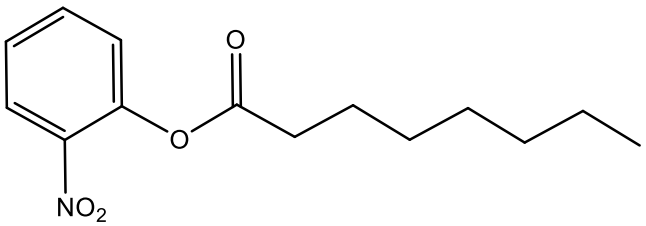
2,6 dimethyl phenyl octanoate



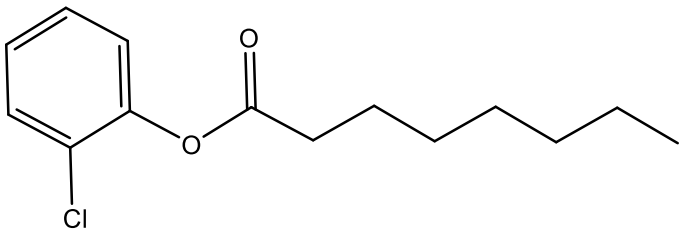
2-methylphenyl octanoate



2-Chloro-4-methylphenyl octanoate



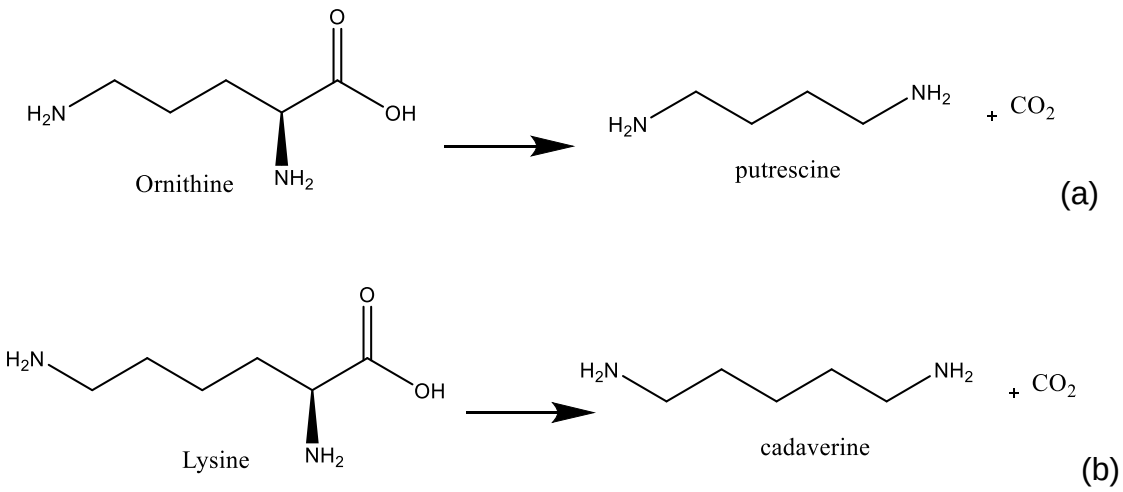
2-nitrophenyl octanoate



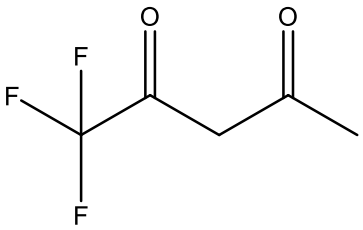
2-chlorophenyl octanoate

Figure 3.3 Structures of evaluated C-8 esterase substrates

The analysis of putrescine and cadaverine cannot be performed in GC without derivatization therefore, trifluoroacetylacetone (TFAA) (Figure 3.4) was the derivative reagent used as part of putrescine and cadaverine sample preparation for GC analysis. The derivatives experiments were conducted on standard solutions in pure broth media and then were applied to *Salmonella* samples. The derivatization approach adopted here is similar to a study described previously by Awan *et al*. (2008).



Scheme 3-1 Decarboxylation of (a) ornithine and (b) lysine



Trifluoroacetylacetone (TFAA)

Figure 3.4 Structure of Trifluoroacetylacetone (TFAA)

3.9.2.5 Non-aqueous/ Organic phase derivatization

The organic phase derivatization was performed to identify and know the retention time and the mass spectra of putrescine and cadaverine derivatives. The derivatives were obtained by reacting a mixture containing 0.5 mL of either putrescine (8.77 µg/mL) and/or cadaverine (8.73 µg/mL) stock solutions in ethanol with TFAA (1.6 mL, 38.2 µg/mL in ethanol) in a 20 mL reaction vial. The vial was sealed and heated to 120 ^{o} C in a silicon oil bath for 20 min. The vial was then left to cool down to room temperature and the mixture was diluted twenty-fold in ethanol before analysis on GC–MS using direct injection. The experiment was repeated by using NMP as a replacement for ethanol.

3.9.2.6 Headspace (on-fiber) derivatization

This experiment was carried out to investigate the presence of putrescine and cadaverine derivatives in the headspace during derivatization. The procedure for HS (on-fibre) derivatization/extraction was carried out by pipetting 2 µL of each putrescine (8.77 µg/mL) and/or cadaverine (8.73 µg/mL) and 45 µL of TFAA (38.2 µg/mL), stock solutions in ethanol or NMP into a 20 mL reaction vial. This procedure maintained a TFAA / amine mole ratio of 22.3 : 1 as recommended by Awan *et al*. (2008). The vial was sealed with a screw cap and heated to 120 ^{o}C for 20 min to evaporating the vial's contents. Derivatization and extraction were occurred at the same time in the headspace. The products were extracted onto the SPME fibre from the vapour phase and desorbed into the injector of the GC–MS system for analyzing. Investigation to derivatization reaction in aqueous phase was carried out using 1 mL, 5 mL and 10 mL of distilled water, TSB, and RVS broth.

3.9.2.6.1 Aqueous phase derivatization and headspace extraction

TFAA, putrescine and cadaverine stock solutions were prepared with ethanol or NMP at concentrations of 38.2 µg/mL, 8.77 µg/mL and 8.73 µg/mL, respectively. A 1 mL of phosphate buffer pH 7 was pipetted into a 20 mL reaction vial that contained 1 mL / or 5mL / or 10 mL sterile broth (TSB or RVS), followed by the addition of 0.8 mL of the TFAA solution and 0.5 mL of either putrescine or cadaverine solution. This protocol maintained a TFAA: amine mole ratio of 4 : 1 in order to insure 100% derivatization as recommended (Awan, 2008). The vial was sealed and heated to 120 ^{o}C for 20 min to evaporating the vial's contents. Derivatization and extraction occurred at the same time in the headspace and after the products were extracted into the fibre from the vapour phase then desorbed into the injector of the GC–MS system for analyzing. The same experiments were repeated using phosphate buffer pH 10 to study the effect of pH on the derivatization reaction. The experiments were also repeated using a TFAA / amine mole ratio of 22.3 : 1 with both buffer solutions.

3.9.2.6.2 Aqueous phase derivatization and solvent extraction

This experiment was carried out to investigate the presence of putrescine and cadaverine derivatives in the solution (broth) as products of the successful reaction. The steps and the process of the reaction and the analysis were as described in Section 3.9.2.4.3; however, the extraction method here is carried out in a different way. The reaction vial was allowed to cool to room temperature and once cooled the reaction mixture was extracted with 1.5 mL of dichloromethane (DCM) at room temperature. The extract was recovered and transferred into a new vial. The extract was then evaporated to dryness under a stream of nitrogen. The residue was dissolved into 2 mL of ethanol to form a stock solution.

This stock solution was diluted further twenty fold in ethanol before analysis on GC-MS using direct injection.

3.9.2.6.3 Cadaverine and putrescine derivatives in *Salmonella* samples

The detection of cadaverine and putrescine in the headspace of 10 mL spiked broth of pH 12 is described here. Lysine and ornithine decarboxylases are formed only when an organism is cultured in an acid environment in the presence of the specific substrates. Therefore, 100 µg/mL of L-ornithine monohydrochloride and L-lysine hydrochloride were added to 10 mL TSB or RVS broth that inoculated with 1.5 $x10^6$ CFU/mL *Salmonella* strain without adjusting the pH. After overnight incubation the pH of the *Salmonella* sample was adjusted to 12 by addition of certain amount of 1 M NaOH followed by addition of 45 µL of the reagent TFAA (38.2 µg/mL). The sample vial then was heated to 120 ^{o}C for 20 min to evaporate the vial's contents. The derivatization and the extraction (HS-SPME) of the derivatives were occurred at the same time in the headspace during heating followed by analysis on GC/MS.

3.9.2.6.4 Evaluation of Falkow media on *Salmonella* samples

Falkow is the decarboxylase media was first described by Moeller and then was developed by Falkow for identification and differentiation of *Salmonella* (Macfaddin, 1987). Bromocresol purple is one of the ingredients in this media used as an indicator of production of cadaverine and putrescine. The alkaline conditions generated due to cadaverine and putrescine production cause the bromocresol purple indicator to revert to a purple colour. In the case of the organism not producing decarboxylase enzyme, the colour of the medium remains yellow. However, in this experiment bromocresol purple was excluded as it is not needed. Falkow`s lysine decarboxylase modified formula used in this experiment contains peptone or Gelysate (BBL) pancreatic digest of gelatin (5 g),

yeast extract (3 g), glucose (1 g), L-lysine hydrochloride solution (5 g) and L-ornithine monohydrochloride (5 g) in 1 litre. Dissolve the weight quantities in 1 litre deionized water and adjust the pH of this solution to 6.8 ± 0.2. Dispense approximately 10 mL per screw-cap vial (20 mL) and, autoclave the vials at 121ºC for 15 min for sterilization. This solution can be stored at 4-10 ºC in fridge until use. Different Falkow`s lysine decarboxylase broth was prepared and tested by changing the amount of the glucose added (1- 10 g/L).

Salmonella samples were prepared using this broth (10 mL each) and after overnight incubation the derivatization procedure as recommended by Awan *et al*. (2008) was carried out. Then 45-50 µL of TFAA of 2.45 M in ethanol was pipetted and injected to the vial through the cap after adding the pH to 12 with 1M NaOH. The vial was then heated to 120 ºC for 20 min to vaporise the vial's contents. Derivatization and extraction occurred at the same time in the headspace and the products were extracted into the fibre from the vapour phase then desorbed into the injector of the GC–MS system for analysis.

3.10 Evaluation of parameters on *Salmonella* detection method

Other parameters that are useful to study when the method applied to detect *Salmonella* in food samples are; the effect of the amount of organic solvent (NMP) used to prepare the stock solution of the substrates, the length of time required to generate VOCs through enzyme substrates hydrolysis and method sensitivity in terms of initial inoculum can be detected in contaminated food samples.

3.10.1 Effect of N-Methyl-2-pyrrolidone (NMP) on *Salmonella* growth

The solvent NMP is a very strong solubilizing agent that has been used to prepare enzyme substrates used in this study. The growth of *S. Stanley*

(prepared as in Section 3.6.2) was tested and recorded at different NMP concentrations (0.5% to 2.5%) after overnight incubation at 37 ºC based on the detection of VOCs liberated by *S. stanley* enzymatic activity using HS-SPME GC/MS. The substrates used were 100 µg/mL chlorohexyl octanoate and phenyl α-D-galactopyranoside in 10 mL RVS broth.

3.10.2 Time study

A time study was conducted by the preparation of 100 µg/mL of the enzyme substrates phenyl α-D-galactopyranoside, 2-chlorophenyl octanoate, 2-nitrophenyl octanoate and incorporated into 10 mL RVS inoculated with *S. stanley* (10^4 CFU/mL). Samples were incubated at 37 ºC in a water bath and subjected to volatile profiling via HS-SPME-GC-MS after 1-hour incubation. Then the broth was monitored over a 24-hour period. This experiment was carried out in duplicate.

3.10.3 Evaluation of method sensitivity

Serial dilutions were made as in Section 3.6.3 of *S. stanley* to assess the sensitivity of the detection method in terms of initial inoculum size. The initial inouclums prepared were: 1–1.5 x 10^5, 1–1.5 x 10^4, 1–1.5 x 10^3, 1–1.5 x 10^2, 1–1.5 x 10^1, 1–1.5 x 10^0 CFU / mL RVS broth. The *Salmonella* samples were prepared as described in Section 3.6.2 with 100 µg/mL phenyl α-D-galactopyranoside, 2-chlorophenyl octanoate, 2-nitrophenyl octanoate as enzyme substrates. All samples were incubated overnight at 37 ºC.

3.11 Synthesis of enzyme substrates

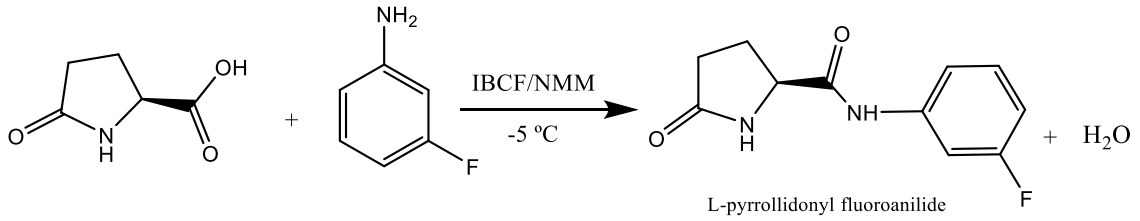
The experiments described in this Section relate to the synthesis of enzyme substrates that release volatile products that can be detected easily using HS-SPME-GC/MS. These experiments seek to provide a unique

identification of *Salmonella* species in food samples and reduce the time taken for the detection. The NMR spectra were obtained on a Jeol 400MHz Eclipse NMR Spectrometer. Low resolution mass spectra (LRMS) were obtained using a Thermo Finnegan Trace GC Ultra and Polaris Q ion trap mass spectrometer. High resolution mass spectrometry (HRMS) was carried out by the EPSRC UK National Mass Spectrometry Facility at Swansea University.

3.11.1 Synthesis of L-pyrrollidonyl fluoroanilide substrate

L-pyrrollidonyl fluoroanilide (Figure 3.2) was prepared following the general procedure described by Cellier *et al*. (2014) in Section 7.1.6 using L-pyroglutamic acid as the amino acid. L-Pyroglutamic acid (1.3 g, 8.99 mmol) was dissolved in dry DMF (20 mL) and cooled to -5 ºC in an ice / salt bath. In a separate flask, to a stirred solution of 3 fluoroaniline (1.1 g, 10.5 mmol) in dry DMF (20 mL) was added N-methylmorpholine (NMM) (1.01 g, 9 mmol)) and the mixture was cooled to -5 ºC. Isobutyl chloroformate (IBCF) (1.4 g, 9 mmol)) was then added to this mixture and stirred for 90 s. After that previously prepared L-pyroglutamic acid solution was added. The resulting mixture was stirred at -5 ºC for 1 h and then at room temperature overnight.

The solvent was evaporated and the residue was dissolved in dichloromethane (DCM). The organic phase was washed sequentially with 0.1 M citric acid solution, 10% aqueous sodium hydrogen carbonate solution and water. The organic layer was dried ($MgSO_4$) and evaporated giving the product as a white solid powder (0.5481g, 80%). Scheme 3.2 shows the reaction and L-pyrrollidonyl fluoroanilide was successfully synthesized and its identity was confirmed by ^1H NMR (Figure 3.5).



Scheme 3-2 Synthesis of L-pyrrollidonyl fluoroanilide

Melting point 182-183.6 °C

^{1}H-NMR (400 MHz; d6-$CDCl_3$) δ; 7.6 (1H, dt, J = 11.45,1.92 Hz, Ar-H), 7.3 (1H, m, Ar-H), 6.85 (1H, m, Ar-H), 4.13 (1H, q, J = 4.23 Hz, CH), 2.12 (4 H, m, 2 x CH_2)

3.11.2 Synthesis of C-8 esterase substrates

As it is well known that C-8 esterase activity is an excellent diagnostic marker for the discrimination of *Salmonella* (+) from most other bacteria (-) (Aguirre *et al*., 1990), some C-8 esterase substrates that could release exogenous VOCs were synthesized and tested on *Salmonella* strains.

3.11.2.1 6-Chlorohexyl octanoate

To a stirred solution of 6-chlorohexanol (0.42 g, 3.1 mmol) and triethylamine (Et_3N) (0.94 g, 9.3 mmol) in dry dichloromethane (DCM) (20 mL) at room temperature was added a solution of octanoyl chloride (0.5 g, 3.1 mmol) in dry dichloromethane (20 mL) drop-wise over 30 minutes. The mixture was stirred (20 h) at room temperature and then dilute aqueous hydrochloric acid (1-2 mL, 4M) was added to neutralize the solution (pH 7-8). The organic layer was separated and the aqueous layer was extracted with dichloromethane (2 x 40 mL). The combined organic extracts were dried ($MgSO_4$) and evaporated yielding yellowish oil (1 g). Scheme 3.3 shows the reaction.

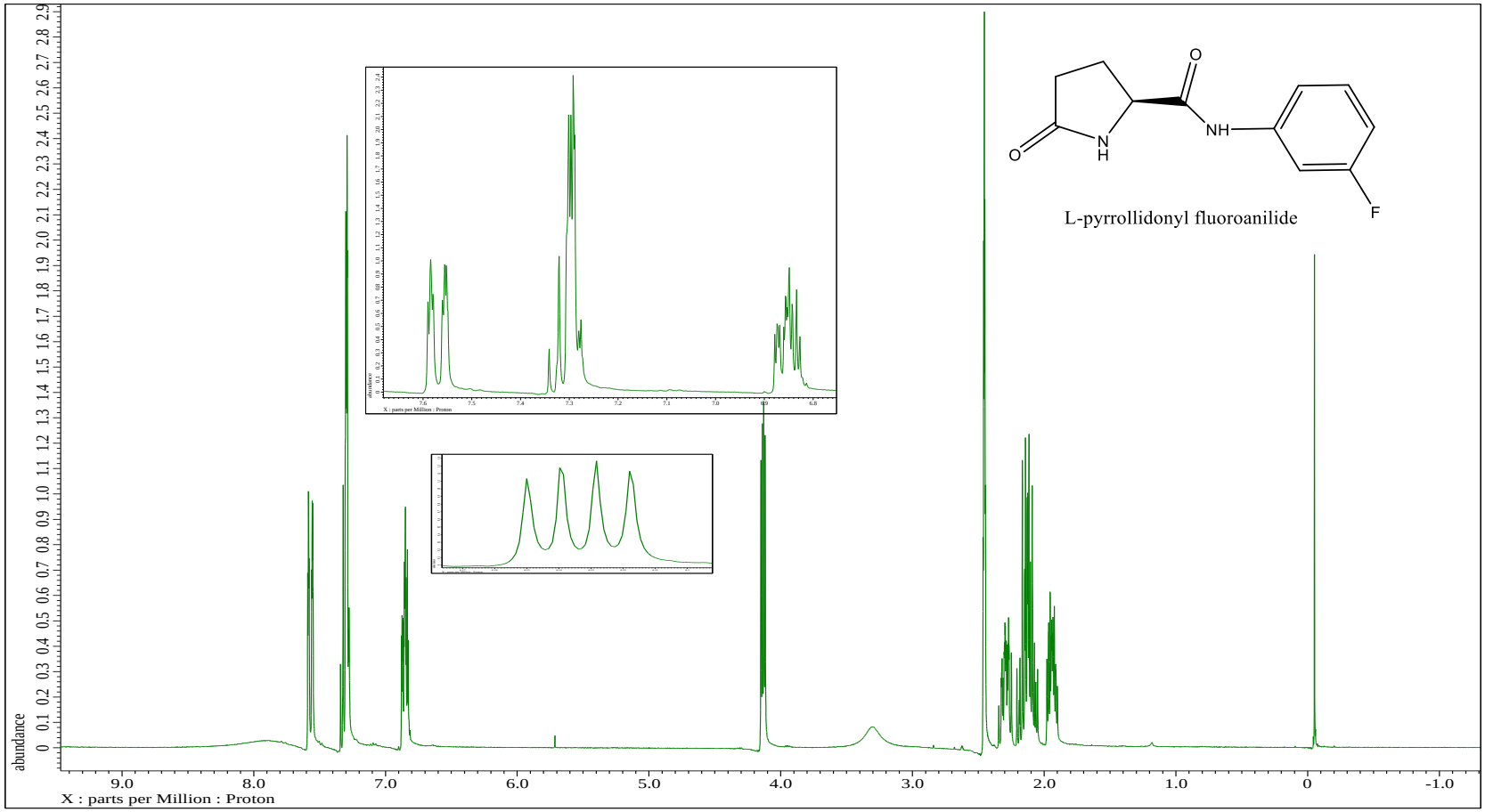
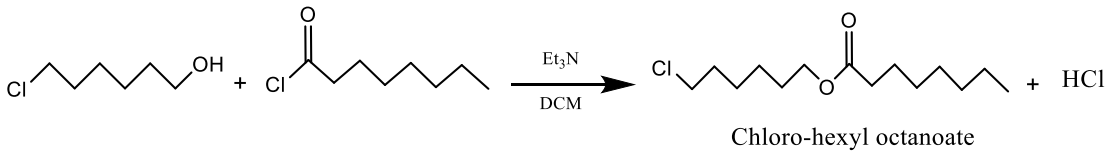


Figure 3.5 ^{1}H-NMR of L-pyrrollidonyl fluoroanilide

The product was purified by vacuum distillation (170 -190 °C, 4 mmHg) and was separated into two layers, a yellow top layer and a white bottom layer and residual (brownish oil) (0.6583 g, 81 %). Residue was the 6-chlorohexyl octanoate substrate as the ^{1}H NMR (Figure 3.6) and the low resolution mass spectrometry (Figure 3.7) confirmed that.



Scheme 3-3 Synthesis of 6-chloro-hexyl octanoate

^{1}H-NMR (400 MHz; $CDCl_3$) δ: 4.07(2H, t, J = 6.64 Hz, CH_2), 3.51 (2H, t, J = 6.64 Hz, CH_2), 2.27 (2H, t, J = 7.57 Hz, CH_2), 1.76 (2H, quin, J = 6.99 Hz, CH_2), 1.66-1.56 (4H, m, 2 x CH_2), 1.49-1.19 (12H, m, 6 CH_2), 0.86 (3 H, t, J = 6.63 Hz, CH_3).

The GC/MS spectra (Figure 3.7) of the synthesized chlorohexyl octanoate indicated that the successful synthesis of this substrate. The molecular ions (M+ and M+2) at 263 and 265, respectively are clearly separated by 2 m/z units with a ratio of 3 : 1 in the peak heights, that states the compound contains 1 chlorine atom and the chlorine can be either of the two chlorine isotopes, ^{35}Cl and ^{37}Cl.

3.11.2.2 Trifluoroethyl octanoate (TFEO)

To a stirred solution of octanoyl chloride (1.08 g, 6.15 mmol) and triethyl amine (0.93 g, 9.23 mmol) in dry DCM (40 mL), 2,2,2-trifluoroethanol (0.628 g, 6.15 mmol) was added drop wise at 0 °C.

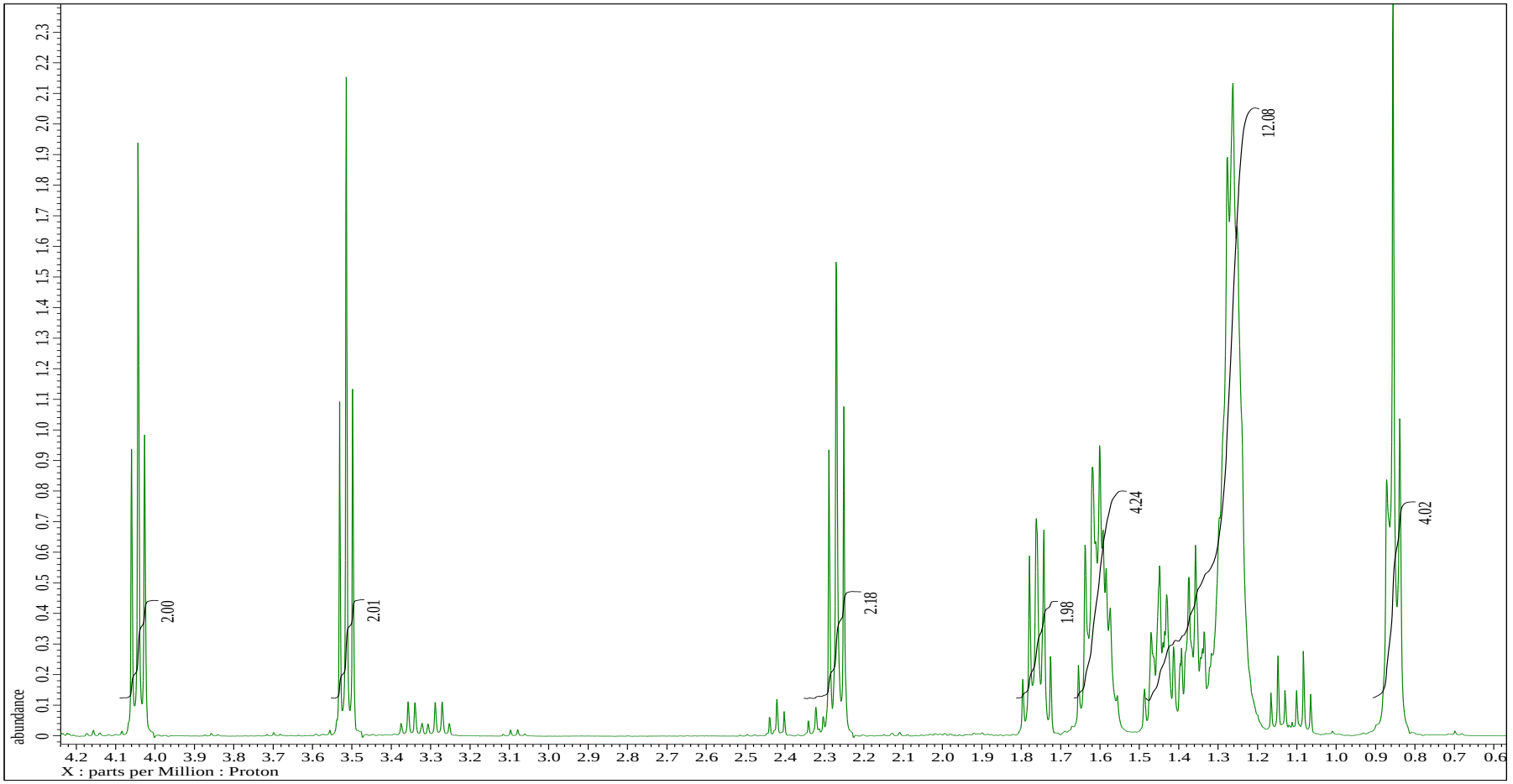


Figure 3.6 ^{1}H-NMR of 6-chlorohexyl octanoate

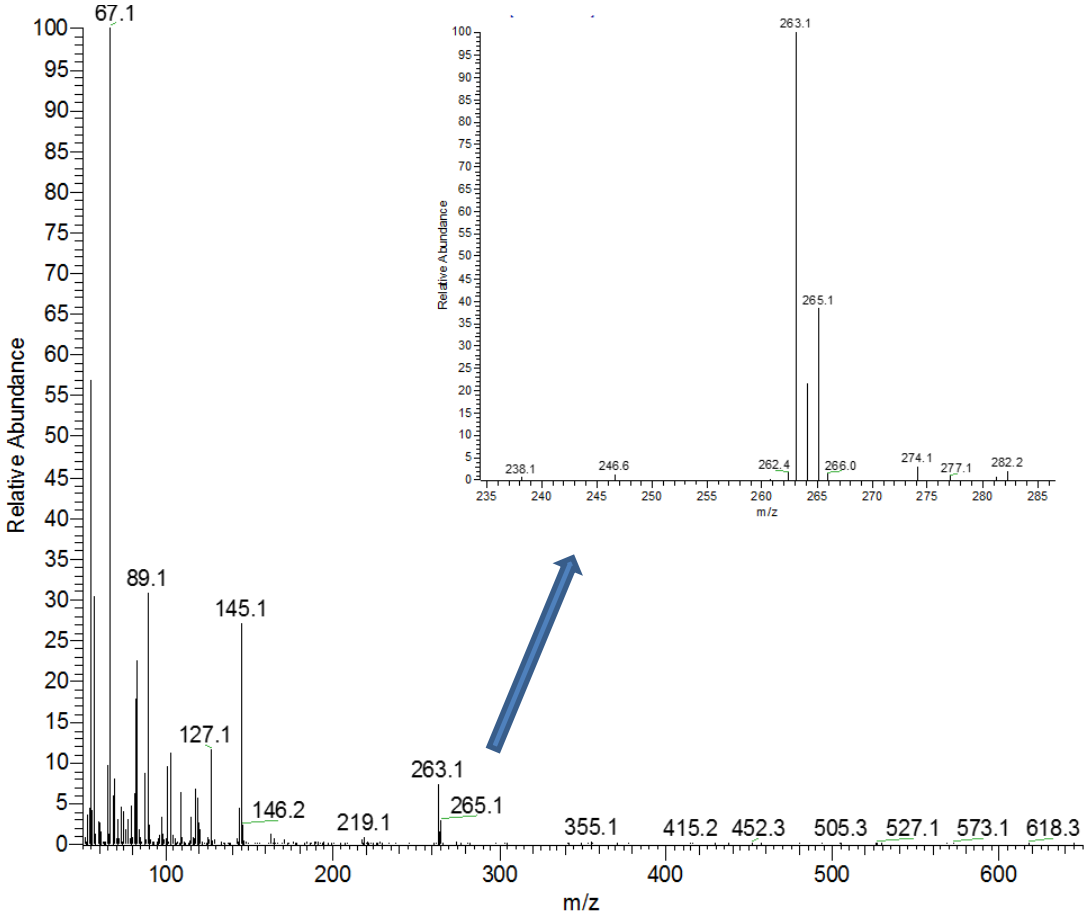
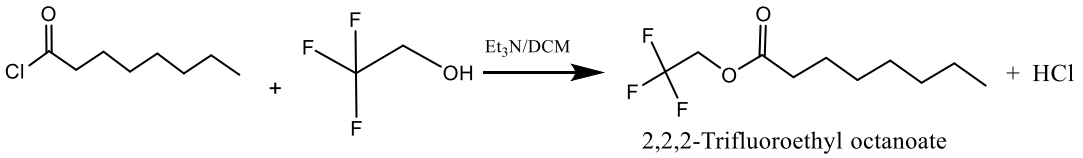


Figure 3.7 Mass spectrum of synthesized chlorohexyl octanoate analyzed with polar GC column and polar SPME fiber

The mixture was stirred at that temperature for one hour then at room temperature for another an hour. The mixture was dried with MgSO4 and the solvent was evaporated giving the product as oil (0.5413 g, 80%). Scheme 3.4 shows the reaction steps. The NMR data (Figure 3.8) confirm the identity of the synthesized TFEO.



Scheme 3-4 Synthesis of 2,2,2-Trifluoroethyl octanoate

^{1}H-NMR (400 MHz; $CDCl_3$) δ; 4.54-4.4 (2H, m, CH_2), 2.45-2.36 (2H, m, 1 x CH_2), 1.48-1.18 (10H, m, 5 x CH_3), 0.86 (3H, t, J = 6.56 Hz, CH_3).

3.11.2.3 Phenyl octanoate substrates

It is as shown in Scheme 3.5, 2,6 dimethyl phenyl octanoate (a), 2-chloro-4-methylphenyl octanoate (b), 2-methyl phenyl octanoate (c), 2-chlorophenyl octanoate (d), and 2-nitrophenyl octanoate (e) were successfully synthesized using the following procedure:

A solution of octanoyl chloride (1 equivalent) in dry dichloromethane (DCM) (20 mL) was added drop-wise to a stirred solution of the appropriate phenolic compound (2,6 dimethyl phenol, 2-chloro-4-methylphenol, 2-methyl phenol, 2-chlorophenol) (1 equivalent) in DCM (20 mL) and triethylamine (2 equiv.) in DCM (20 mL).

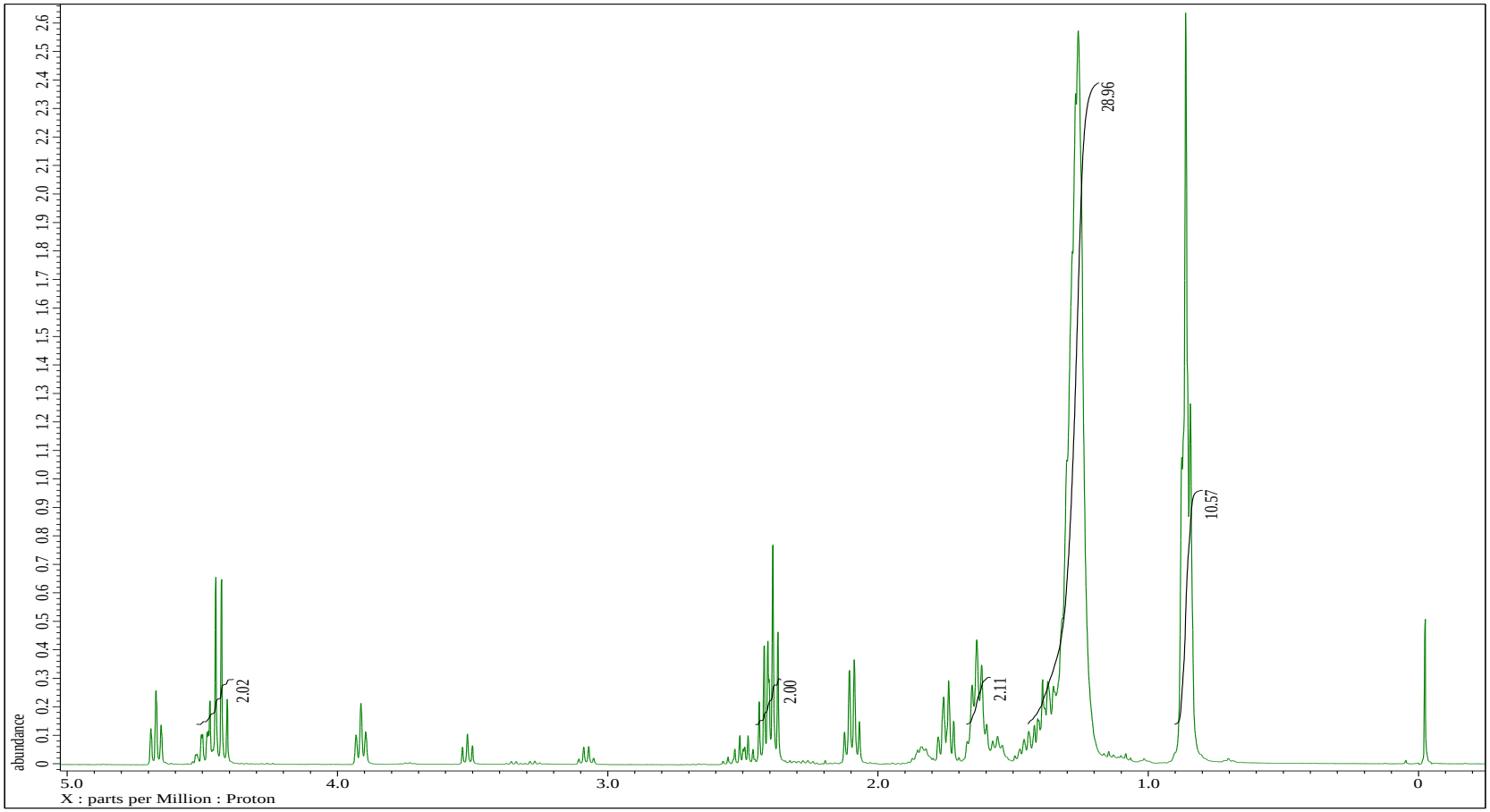
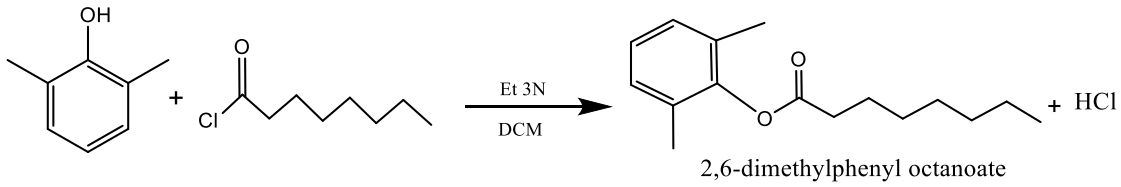


Figure 3.8 ^{1}H-NMR of 2,2,2-Trifluoroethyl octanoate

The mixture was allowed to stir at room temperature for 20 h and then neutralized (pH 7-8) by the addition of dilute aqueous HCl (1 M). Water (20 mL) was then added and the mixture was extracted using dichloromethane (2 x 20 mL) and the organic extracts were dried using $MgSO_4$. The solvent (DCM) was evaporated giving an oily residue which was the required phenolic ester. The product in each experiment was purified by vacuum distillation (160 -190 °C, 4 mmHg), and the oily residue was the desired substrate and the analytical data of the products are showing in the following:

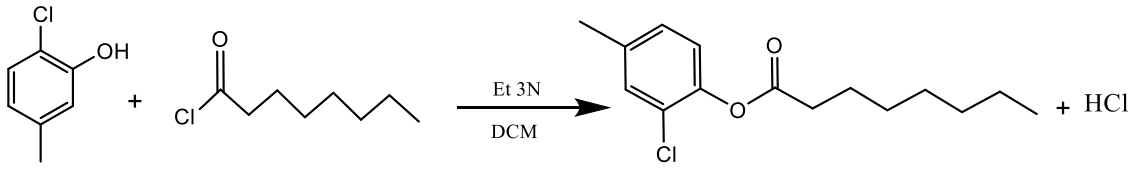
2,6 Dimethyl phenyl octanoate

2,6 Dimethyl phenyl octanoate (Scheme 3.5 (a)) was successfully synthesised. This substrate has not been previously synthesized, and the ^1H NMR spectrum (Figure 3.9),^{13}C NMR spectrum (Figure 3.10) and HRMS of this substrate have shown the evidence of the successful synthesis.



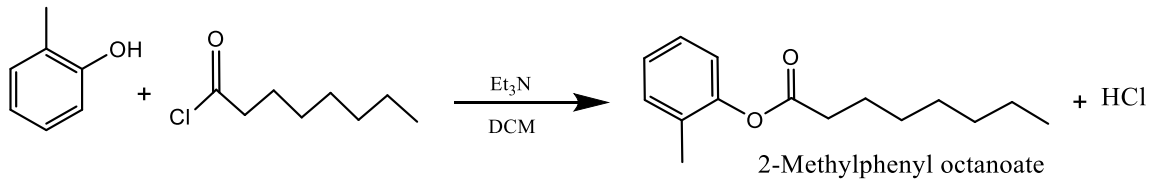
2,6-dimethylphenyl octanoate

(a)

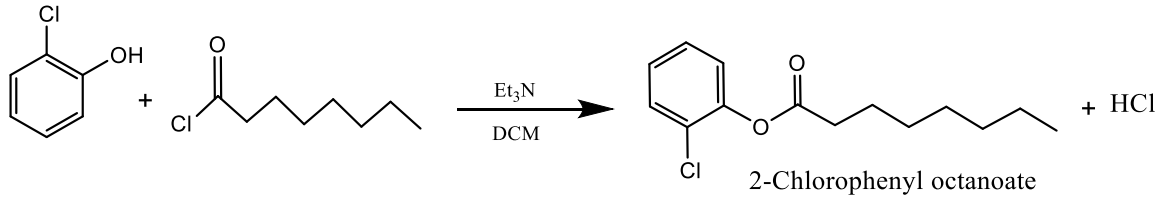


2-Chloro-4-methylphenyl octanoate

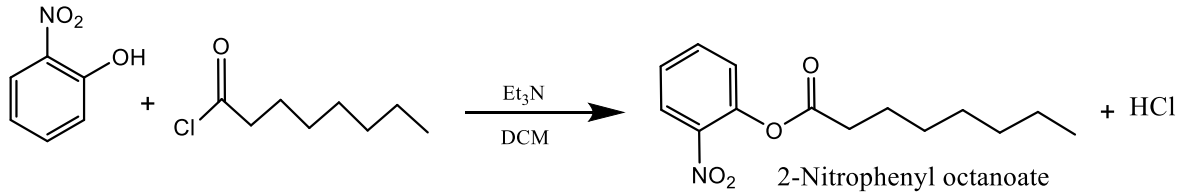
(b)



(c)



(d)



(e)

Scheme 3-5 Synthesis of phenolic C-8 esterase substrate

^{1}H-NMR (400 MHz; $CDCl_3$) δ: 7.05 (3H, m, Ar-H), 2.59 (2 H, t, 7.59 Hz, CH_2), 2.15 (6 H, s, CH_2), 1.79 (2H, p, 15.11, 7.79, 7.59, 7.33 Hz, CH_2), 1.46-1.26 (8 H, m, CH_2), 0.9 (3 H, t, 6.18, 6.87 Hz, CH_3).

^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.6 (C=O), 148.5 (Ar-C), 130.2 (Ar-C), 128.6 (Ar-C), 125.8 (Ar-C), 34.7(CH_2), 31.8(CH_2), 29.4(CH_2), 29(CH_2), 25.3(CH_2), 22.7 (CH_2), 14.2 (3 x CH_3).

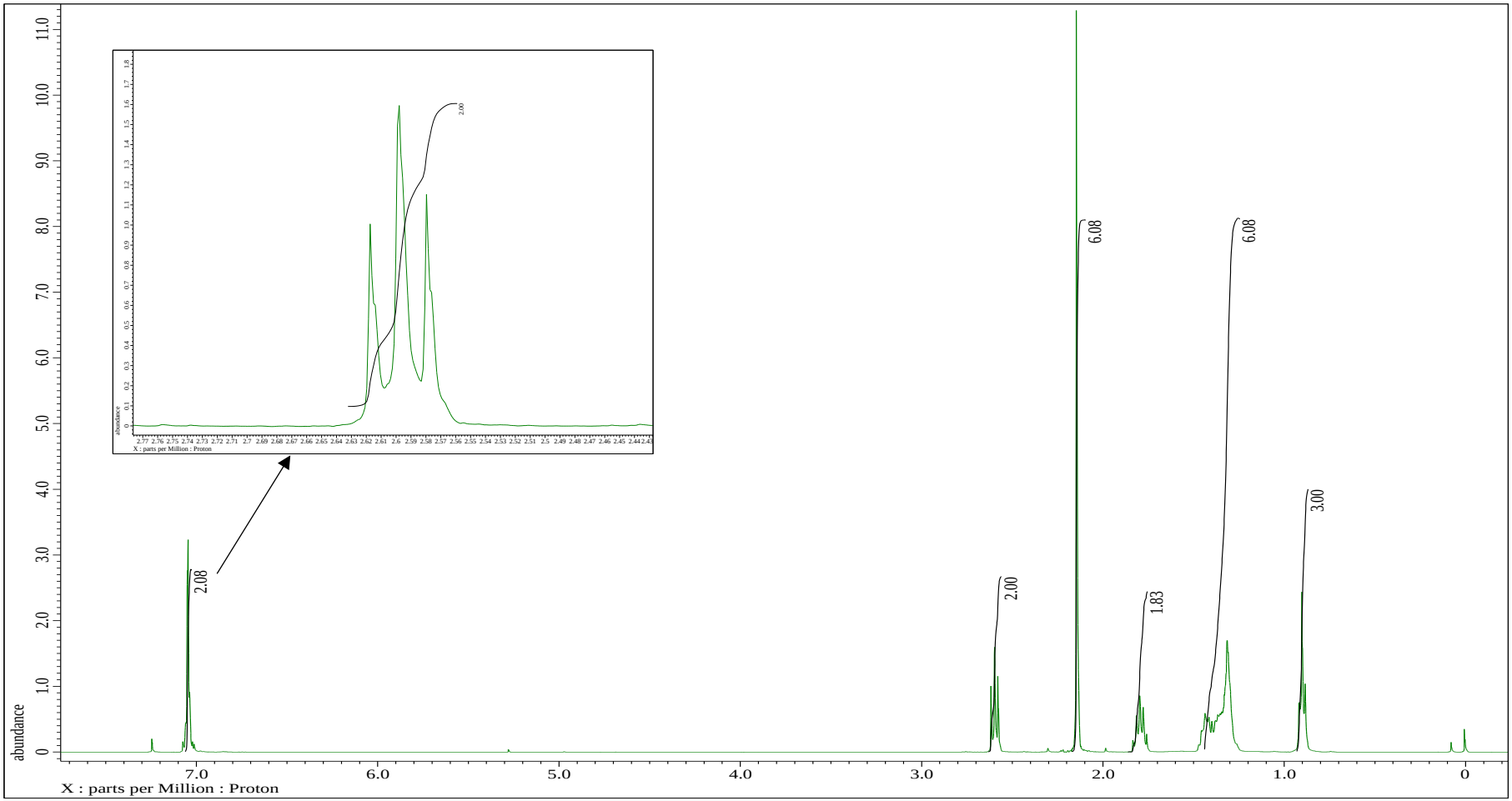


Figure 3.9 ^{1}H-NMR of 2,6 dimethyl phenyl octanoate

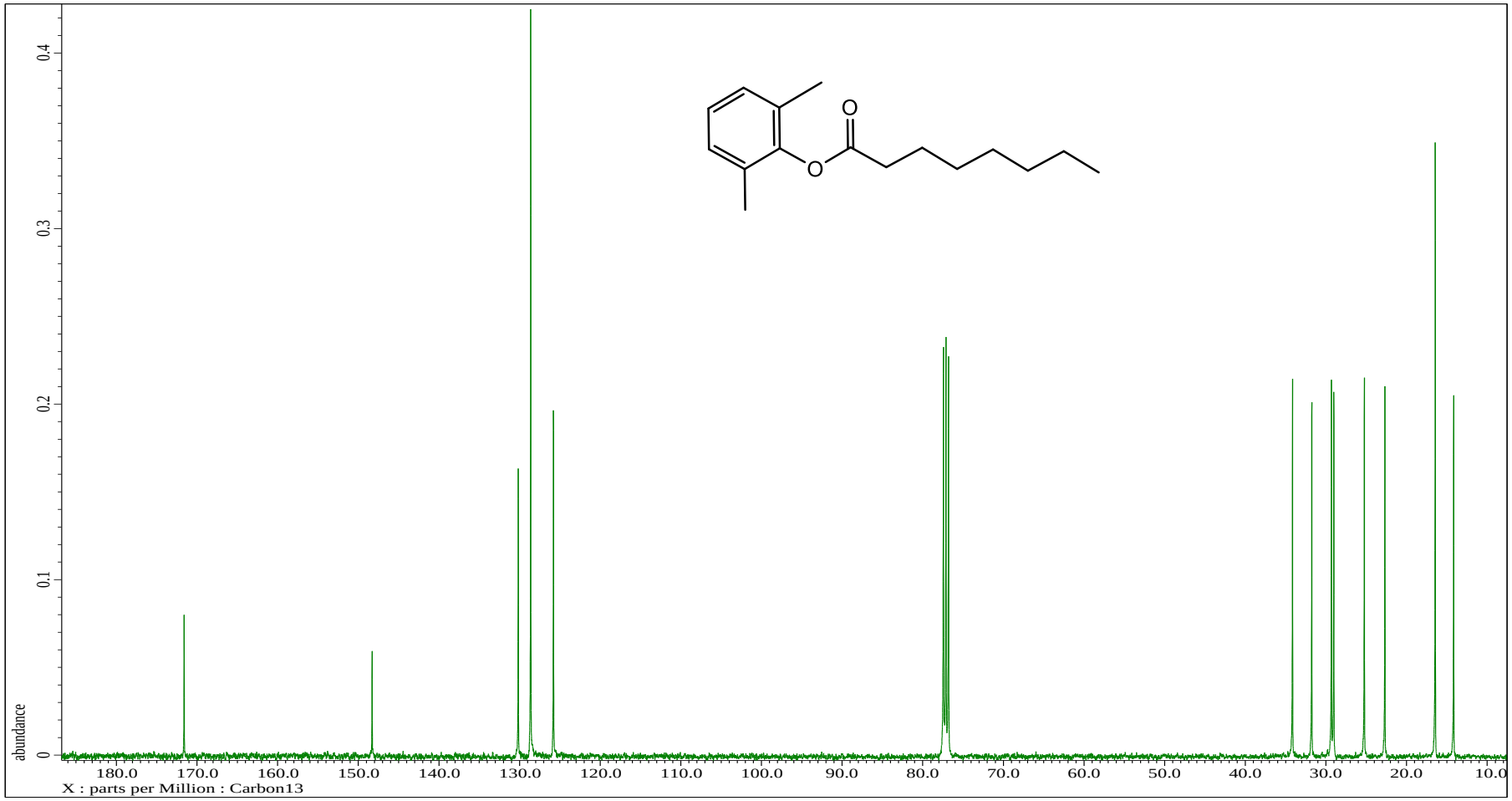


Figure 3.10 ^{13}C NMR spectrum of 2,6 dimethyl phenyl octanoate

2-Chloro-4-methylphenyl octanoate

The substrate 2-chloro-4-methylphenyl octanoate (Scheme 3.5 (b)) was synthesised for the first time correctly and the NMR experiments and the HRMS were performed to prove the correct structure of 2-chloro-4-methylphenyl octanoate. The spectra are shown in Figure 3.11 and 3.12.

^{1}H-NMR (400 MHz; $CDCl_3$) δ: 7.24 (H, d, J = 5.50, Hz, Ar-H), 7.05 (H, dt, 8.93, 1.37, 0.92 Hz, Ar-H), 6.98 (H, d, 8.24 Hz, Ar-H), 2.58 (2H, t, 3.73 Hz, CH_2), 2.31 (3 H, s, Ar-H), 1.78 (2H, p, 7.79, 7.56, 7.33 Hz, CH_2), 1.35 (8 H, m, CH_2), 0.88 (3 H, t, 6.87 Hz, CH_3).

^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.6 (C=O), 144.7 (Ar-C), 137.1 (Ar-C), 130.7(Ar-C), 128.4 (Ar-C), 126.4 (Ar-C), 123.3 (Ar-C), 34.1 (CH_2), 31.7 (CH_2), 29.16 (CH_2), 29 (CH_2), 25.1 (CH_2), 22.7 (CH_2), 20.8 (CH_2), 14.4 (2 x CH_3).

2-Methyl phenyl octanoate

2-Methyl phenyl octanoate (scheme 3.6 (c)) was successfully synthesized as the ^{1}HNMR spectrum (Figure 3.13) shows that.

^{1}H-NMR (400 MHz; $CDCl_3$) δ: 7.21 (2H, p, J = 8.24, 6.87, 5.95 Hz, Ar-H), 7.12 (H, dt, 7.37, 1.37 Hz, Ar-H), 6.98 (H, dd, 8.01, 1.37 Hz, Ar-H), 2.57 (2 H, t 7.79, 7.5 7.3 Hz, CH_2), 2.12 (3 H, s, Ar-CH_3), 1.78 (2H, q, 7.79, 7.56, 7.33 Hz, CH_2), 1.35 (8 H, m, CH_2). 0.89 (3 H, t, 6.87 Hz, CH_3).

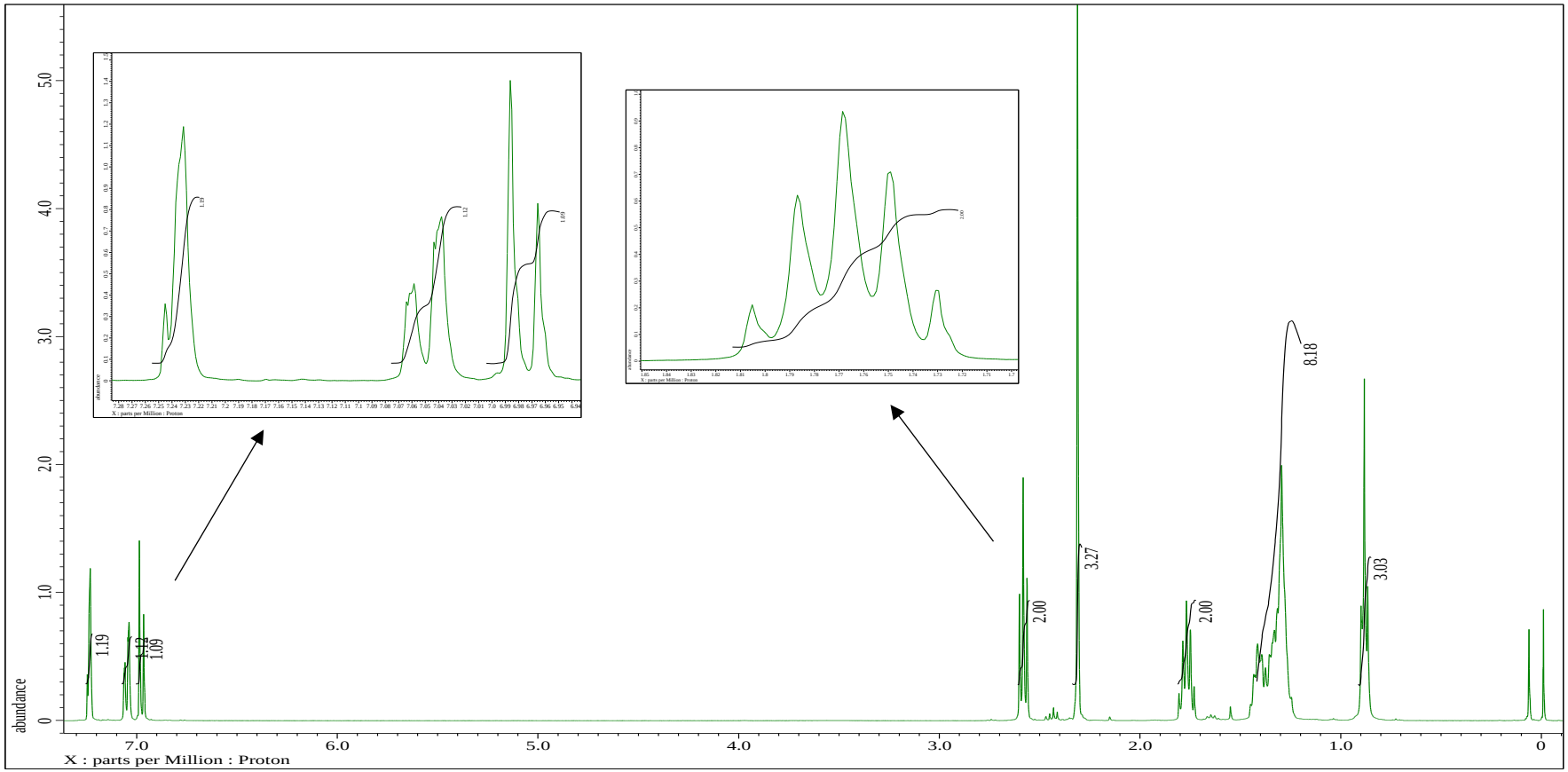


Figure 3.11 ^{1}H-NMR of 2-chloro-4-methylphenyl octanoate

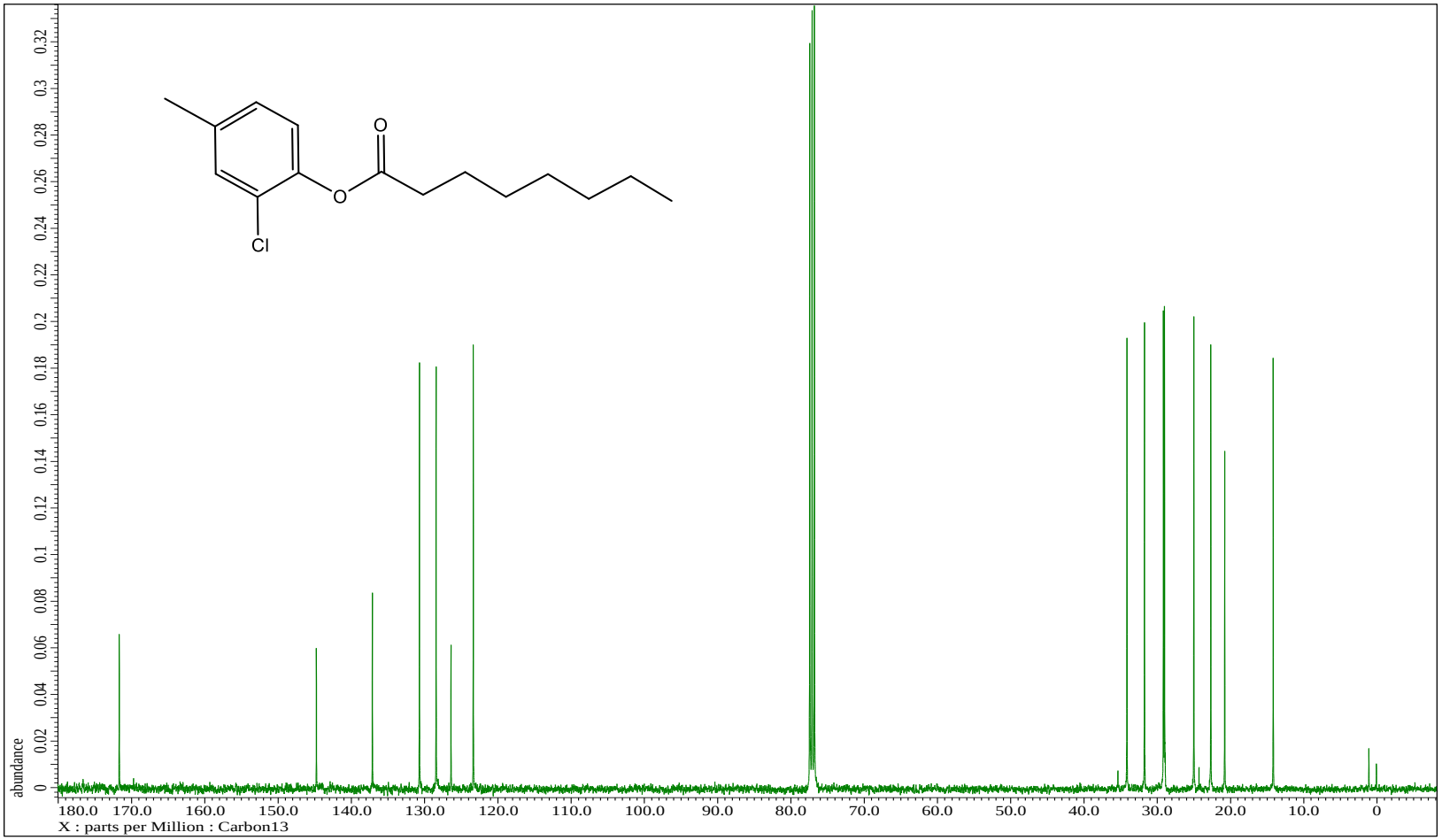


Figure 3.12 ^{13}C NMR spectrum of 2-chloro-4-methylphenyl octanoate

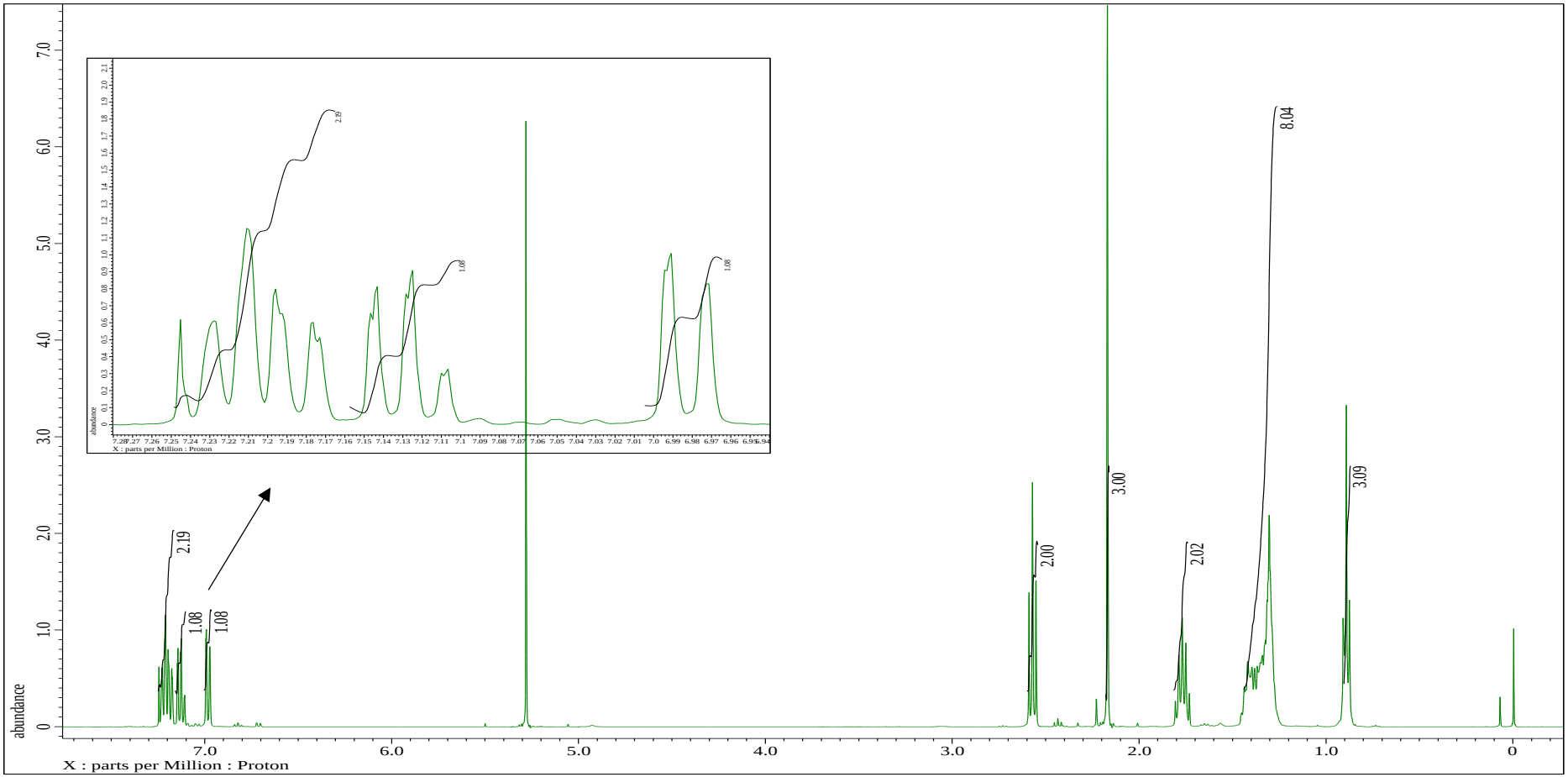


Figure 3.13 ^{1}H-NMR of 2-methyl phenyl octanoate

2-Chlorophenyl octanoate

The proton signals in Figure 3.14 and the chemical shifts detected in ^{13}C NMR spectra Figure 3.15 and the HRMS result (Figure 3.16) indicated the successful synthesis of 2-chlorophenyl octanoate (Scheme 3.6 (d)) for the first time.

^{1}H-NMR (400 MHz; CDCl3) δ: 7.42 (H, dd, J = 8.4, 1.6 Hz, Ar-H), 7.26 (H, td, J = 8.4, 1.6 Hz, Ar-H), 7.17 (H, td, J = 8.4, 1.6 Hz, Ar-H), 7.11 (H, dd, J = 8.4, 1.6 Hz, Ar-H), 2.60 (2H, t, J = 7.2 Hz, CH_2), 1.77 (2H, p, J = 7.2Hz, CH_2), 1.45-1.22 (8 H, m, CH_2), 0.88 (3H, t, J = 7.2 Hz).

^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.5 (C=O), 147.2 (Ar-C), 130.3 (Ar-C), 127.8 (Ar-C), 127.0 (Ar-C), 123.9 (Ar-C), 34.1 (CH_2), 31.7(CH_2), 29.2 (CH_2), 29.0 (CH_2), 24.9 (CH_2), 22.7 (CH_2), 14.2 (CH_3).

HRMS (M + NH_4^+) calculated /found; m/z 272.1412 / m/z 272.1416 (Figure 3.16)

2-Nitrophenyl octanoate

2-Nitrophenyl octanoate (Scheme 3.6 (e)) was synthesised successfully as the signals in the NMR spectra in Figure 3.17 and 3.18 explain that and the HRMS result (Figure 3.19) confirm the identity of the synthesized compound.

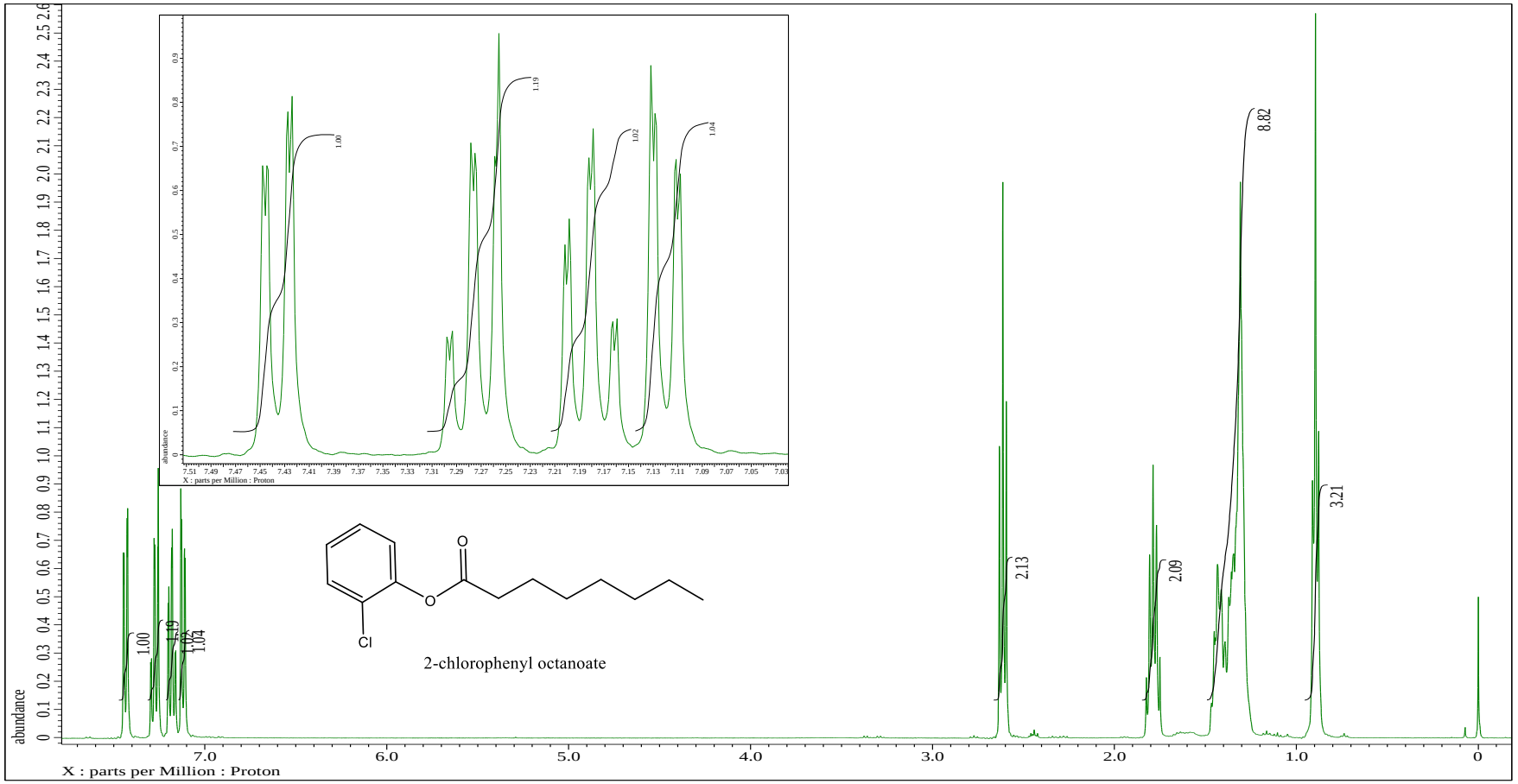


Figure 3.14 ^{1}H-NMR of 2-chlorophenyl octanoate

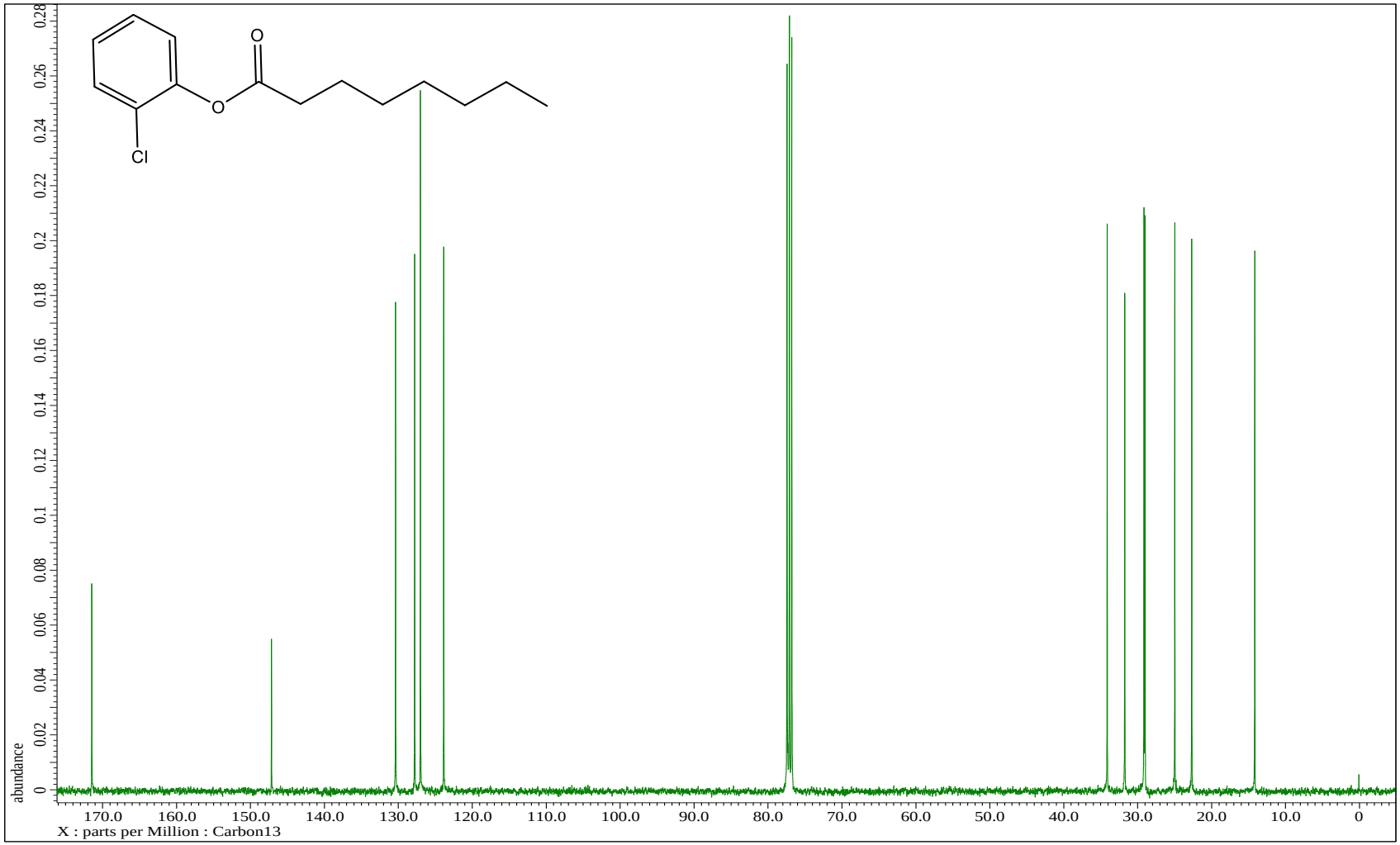


Figure 3.15 ^{13}C-NMR of 2-chlorophenyl octanoate

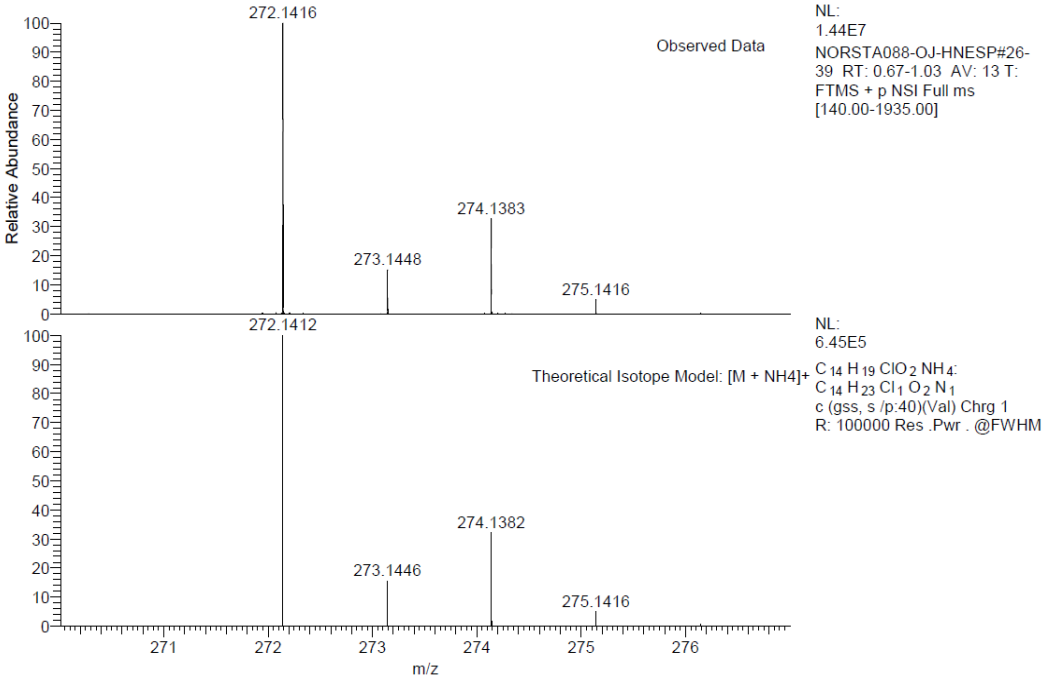


Figure 3.16 High-resolution mass spectrum of 2-chlorophenyl octanoate

^{1}H-NMR (400 MHz; $CDCl_3$) δ: 8.07 (H, dd, 1.8, 8.24 Hz, Ar-H), 7.63 (H, td, 8.01, 7.46, 1.83, 1.37 Hz, Ar-H), 7.37 (H, td, 8.24, 7.56, 1.37, 0.92 Hz, Ar-H), 7.22 (10.07, 8.47,6.87 Hz, Ar-H), 5.27 (2 H, s, CH_2), 2.62 (2H, t, 7.79, 7.56, 7.33, Hz, CH_2), 1.45-1.21 (8H, m, CH_2), 0.88 (3 H, t, 6.87 Hz, CH_3).

^{13}C NMR (100 MHz; $CDCl_3$) δ: 171.5 (C=O), 144.3 (Ar-C), 134.8 (Ar-C), 126.6 (Ar-C), 125.9 (Ar-C), 125.4 (Ar-C), 34.2 (CH_2), 31.7 (CH_2), 29.1 (CH_2), 29 (CH_2), 24.8, 22.7 (CH_2), 14.2 (CH_3).

HRMS (M + NH_4^+) calculated /found; m/z 283.1652 / m/z 283.1651 (Figure 3.19)

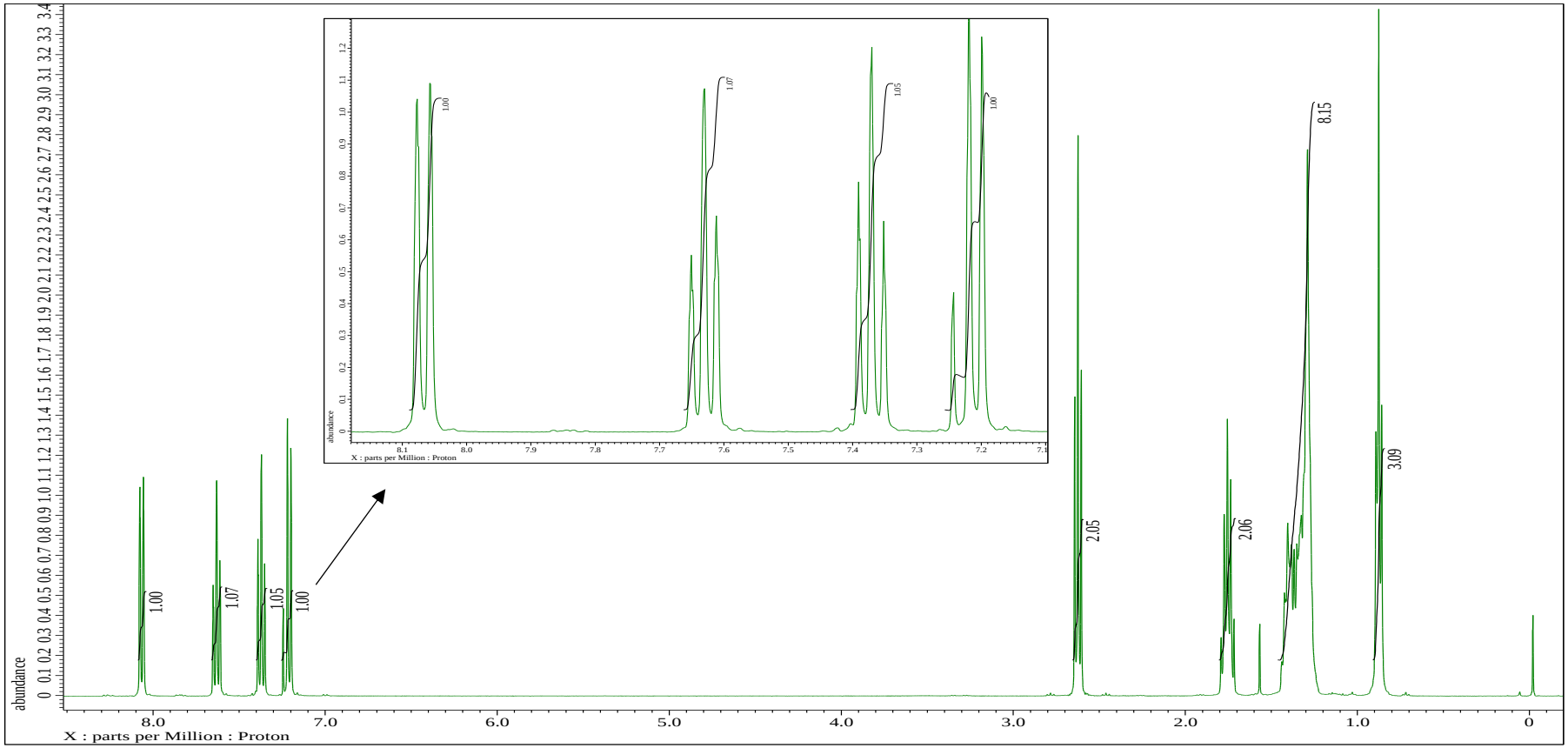


Figure 3.17 ^{1}H-NMR of 2-nitrophenyl octanoate

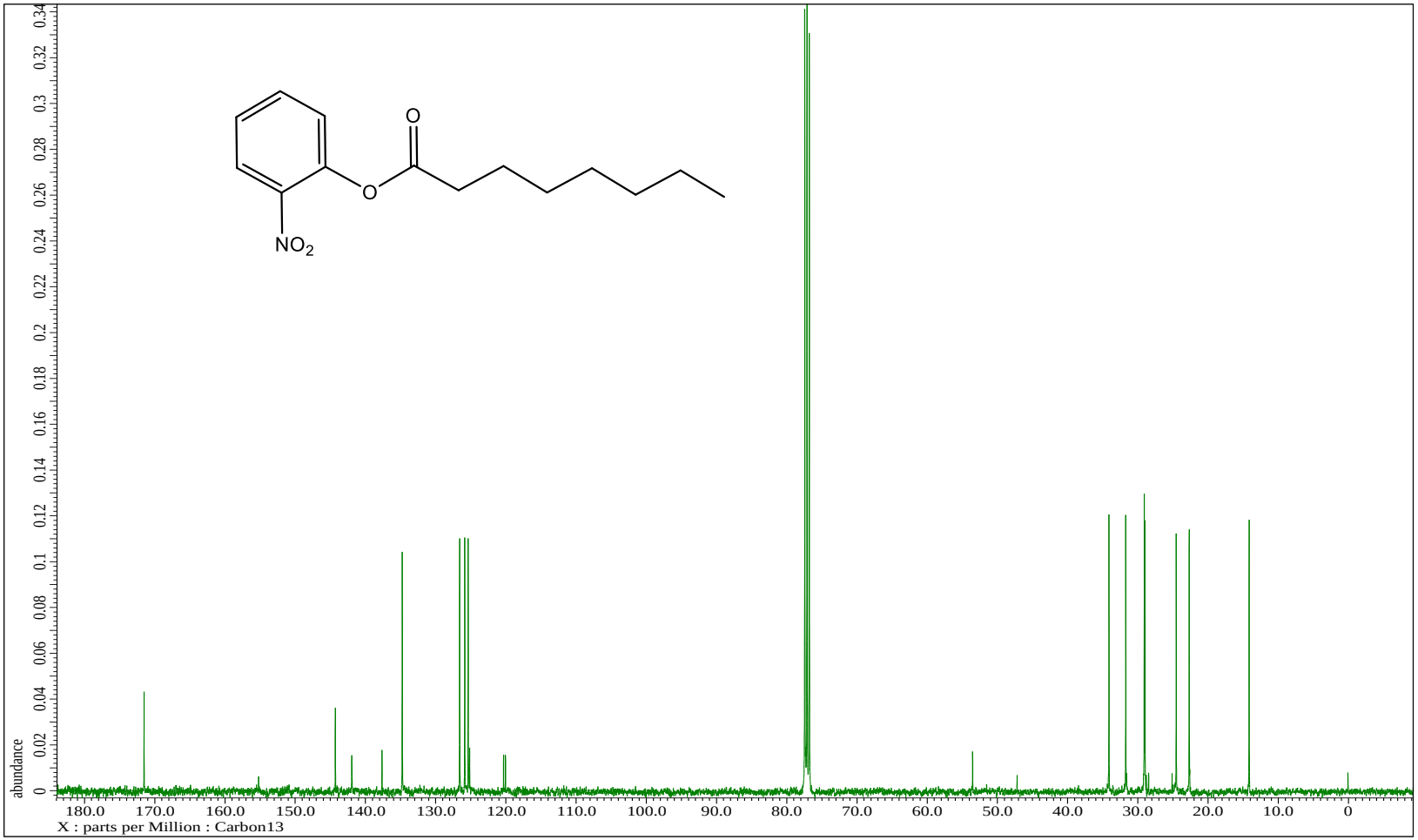


Figure 3.18 ^{13}C NMR spectrum of 2-nitrophenyl octanoate

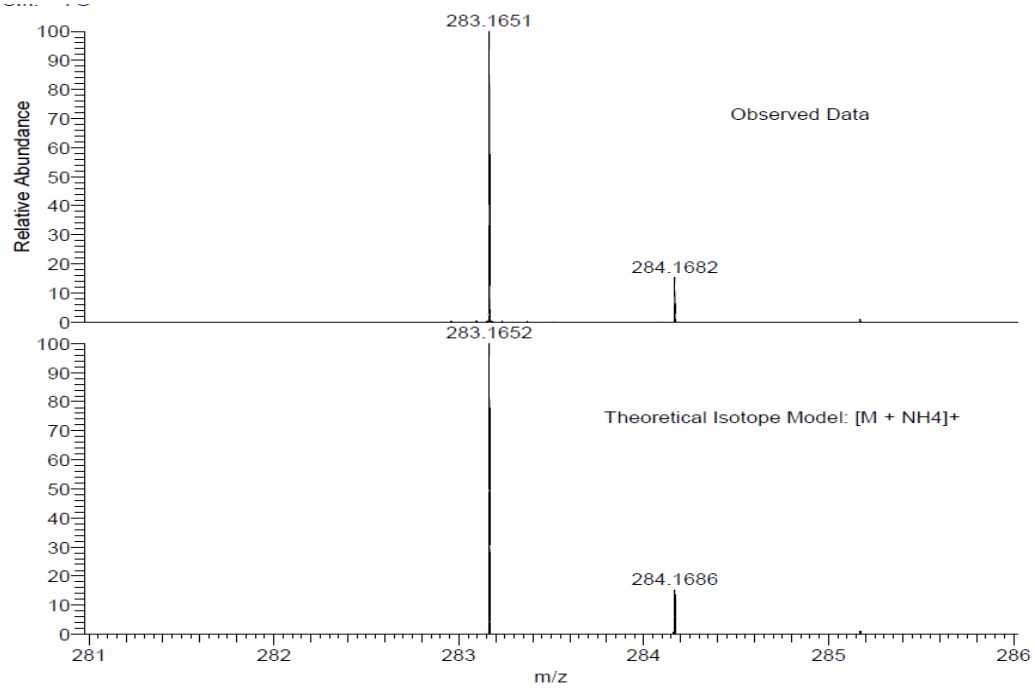


Figure 3.19 High resolution mass spectrum of 2-nitrophenyl octanoate

3.12 Food analysis

Application of the proposed analytical procedures for detecting *Salmonell*a in food samples were carried out using milk, cheese, eggs and raw chicken samples as outlined in the next Sections.

3.12.1 Food samples preparation

Food samples were prepared in such a way to ensure that no bacterial contamination was introduced by contact with hands or contact with unsterile surfaces or items. Detection of *Salmonella* in foods involved pre-enrichment of the food sample in a nonselective broth to allow recovery of injured cells and growth of the organisms this method (ISO 6579:2002), followed by incubation on selective enrichment broth, isolation and extraction of target VOCs using HS-SPME followed by separation and identification by GC/MS. Five grams of each sample were placed into a sterile stomacher tube containing 45 mL sterilized

buffered peptone water. The food samples were homogenized and incubated at 37 °C for 16 to 20 h using MPB 1500 water-bath rotator with precise temperature control. After the non-selective pre-enrichment step 1 mL of food sample was inoculated on 9 mL RVS (the selective enrichment broth) which contained a 100 µg/mL of the three enzyme substrates; L-pyrrollidonyl fluoroanilide, 2-chlorophenyl octanoate, and phenyl α-D-galactopyranoside. Spiked food samples were prepared in the same manner as un-spiked ones and with addition 100 µL of *Salmonella* stanley (1x10 ^{6} CFU/mL) to each food sample. Un-spiked 1 mL BPW in 9 mL RVS contains 100 µg/mL of the three enzyme substrates; L-pyrrollidonyl fluoroanilide, 2-chlorophenyl octanoate, and Phenyl α-D-galactopyranoside were used as negative control and blank. These spiked and un-spiked food samples and the control blank were then incubated for 18-24 h at 37 °C. Sampling of VOCs released by bacteria present in the food samples and identification of these VOCs were performed as described in Sections 3.7 and 3.8.1, respectively. All food samples (spiked and un-spiked) were run in triplicate.

3.12.2 Food samples preparation with antibiotic

In this experiment preparation of spiked and un-spiked food samples were prepared as described in Section 3.11.1 with addition of vancomycin (5 mg/L) and novobiocin (10 mg/L) in water to the vials before the food samples were incubated. All the sampling and analyzing steps were as in Section 3.11.1. A cheese sample was studied with addition of vancomycin (5 mg/L) and novobiocin (10 mg/L) two times, in homogenization step and in incubation step.

3.12.3 Identification of bacteria isolated from food samples

After VOCs analysis on the food samples, the samples were incorporated into a nutrient medium by subculture them into *Salmonella* ABC and CLED agar

plates. The plates were overnight incubated at 37°C and the growing bacteria were isolated and colonies were identified to species level using Matrix Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI–TOF MS). A single bacterial colony of each isolate was picked and deposited on a MALDI target plate position with care. The sample spot (after they had been dried) were overlaid with 1 µL Bruker HCCA matrix solution. HCCA matrix solution must be added within 30 minutes after sample spots were dried, or these positions cannot be tested. The matrix-overlaid sample spot was allowed to dry at room temperature and a homogeneous preparation were observed. Then MALDI-TOF-MS measurement was performed.

3.12.4 Evaluation of esterase activities of antibiotic-resistant bacteria

Seven species of different bacteria representative of the antibiotic-resistant bacteria isolated from food samples tested were provided on blood culture agar plates by Professor John Perry at the Microbiology Department, Freeman Hospital Newcastle upon Tyne. These species include *Enterobacter cloacae* (NCTC 11936), *Enterococcus faecalis* (NCTC 775), *Enterococcus faecium* (NCTC 7171), *Streptococcus salivarius* (NCTC 8618), *Cronobacter sakazakii* (ATCC 29544), *Klebsiella oxytoca* (Wild strain), *Serratia marcescens* (NCTC 10211) – very closely related to *Serratia rubidaea* found in food samples. The esterase activity was investigated using two enzyme substrates. Firstly, the bacteria were tested using 2-chlorophenyl octanoate and HS-SPME GC/MS analysis. Secondly, test was performed using the fluorogenic substrate 4-methylumbelliferyl caprylate (Figure 3.19)

3.12.4.1 HS-SPME-GC / MS analysis

The bacteria were tested using 2-chlorophenyl octanoate and HS-SPME

GC/MS analysis. The preparation of bacteria suspension and bacterial samples were as indicated in Section 3.6.1 and 3.6.2. Sampling and analysis of the VOC were as described in Section 3.7 and 3.8.1, respectively.

3.12.4.2 Fluorescent study

The study was performed using the fluoroginic substrate 4-methylumbelliferyl caprylate (Figure 3.19). 4-Methylumbelliferyl caprylate substrate stock solution was prepared in NMP at a concentration of 100,000 µg/mL. Samples of bacteria were prepared as described in Section 3.6.1 and 3.6.2. Then, 100 µg/mL of this substrate were added to the vial of 10 mL RVS broth containing 0.075 g Tween 20 before adding bacteria suspension on (1.5 x 10^8 CFUs). After overnight incubation at 37 °C, the content of the vials was centrifuged at 20,000 x g for 10 minutes to remove the bacterial cells using MSE Harrier 18/80R Refrigerated Benchtop Centrifuge. After removal of bacterial cells from suspensions, a 5 mL were transferred to another sterile vial and the pH of the solution was measured. Bacteria produce acid as they grow because of that, the pH was adjusted to 7.5 using 1 M NaOH as the addition of alkali to the organism-4-methylumbelliferyl substrate complexes after incubation worked well in several quantitative and qualitative investigations (Bobey and Ederer, 1981; Grange and Clark, 1977; Maddocks and Greenan, 1975). These vials were then inspected for the presence (or absence) of fluorescence. An un-inoculated RVS broth contains same quantities of the substrate, Tween 20 and NaOH solution were used as a control.

The calibration curves were constructed with concentrations and intensity responses for quantitative determination of the produced fluorescent using external calibration. Fluorescent studies were carried using FluoroMax ®-4 and

FluoroMax ®-4 Pspectro-fluorometer (HORIBA Jobin Yvon Inc) for screening the samples for presence of the fluorescent MUF (Figure 3.19).

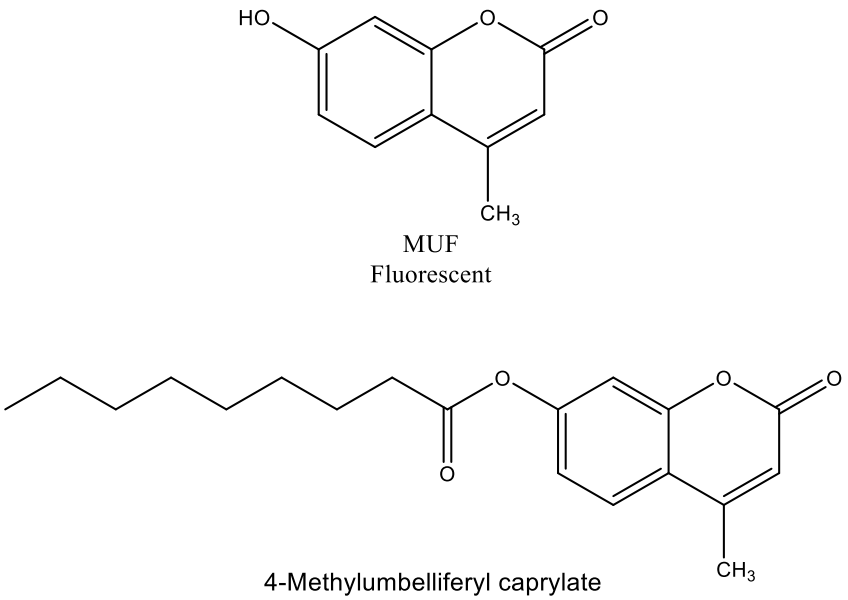


Figure 3.20 Structure of MUF and 4-methylumbelliferyl caprylate

3.13 Summary

Description to the chemicals, methods and techniques used for developing a method for detection and identification of the *Salmonella* based on detection of metabolites' VOCs has been given in this chapter. Synthesis method of some enzyme substrates was outlines with their obtained analytical data. The analysis method applied to study the *Salmonella* VOCs with evaluation to the chosen enzymatic activities was described. Application of the developed detection method onto food samples was outlined with attempts to translate the VOCs fingerprint of *Salmonella* to an optical detection method for ease the application of the developed detection method are also described here.

Chapter 4: Study of the VOC profiles associated with *Salmonella* strains by HS-SPME-GC-MS

4.1 Introduction

It is unlikely that a single VOC could act as a marker for a specific bacterial species. It was therefore envisaged that a number of VOC markers or entire VOC profiles would be more effective in enabling identification and differentiation between bacteria strains. Preliminary investigations were first carried out to identify headspace VOCs associated with nutrient broths spiked with *Salmonella* strains. HS-SPME coupled to GC–MS was applied to the analysis of *Salmonella* VOCs using 6 strains of *Salmonella* inoculated in sterile BHI, TSB and RVS broths. The extraction conditions and analysis method done in this study was recommended by previous studies (Tait *et al*., 2013). The VOCs were quantified using an external calibration method as recommended by Brevard (2010). The effect of culture medium; SPME fiber type; and, GC column polarity were evaluated using 6 strains of *Salmonella*. The HS-SPME-GC-MS data were investigated statistically using principal component analysis to determine whether the parameters under investigation significantly affected *Salmonella* VOC profiles.

4.2 Identification and quantification of *Salmonella* VOCs

Salmonella VOCs were extracted from the headspace of inoculated broths via SPME and inserted in the hot GC injection port. The separated unknowns generated by *Salmonella* strains were identified by comparing their retention times and mass spectra with authentic standards. For instance, the generated unknowns by *S. gallinarum* (Figure 4.1) were identified by comparing their retention times and mass spectra with accurate standards.

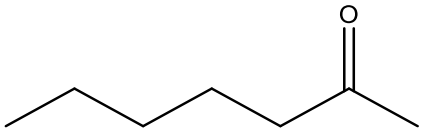
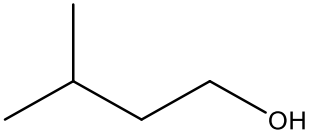
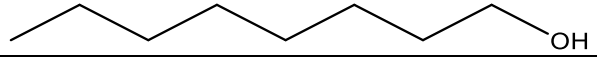
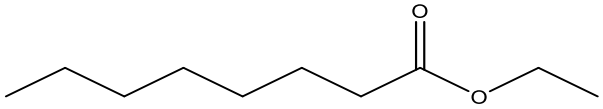
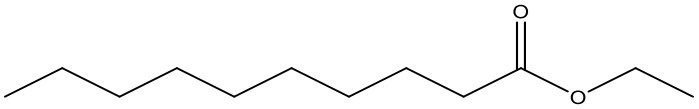
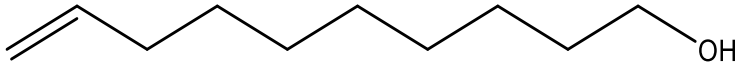
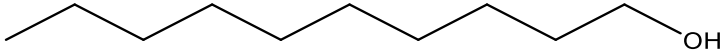
For example, the compound that is liberated by *S. gallinarum* inoculated in TSB, extracted with a PDMS SPME fiber, analyzed with a non-polar GC column

and detected at a retention time of 11.7 minutes (Figure 4.1) was found to be 1-decanol. This is based on its retention time as compared to the retention time of standard 1-decanol (Figure 4.2). Also the mass spectrum of the standard 1-decanol (Figure 4.3) was identical to the mass spectrum (Figure 4.4) of the unknown VOC liberated by *gallinarum*. Both mass spectra show the same fragmentation pattren that illustrates the loss of water from the molecular ion of 1-decanol (molecular weight = 158 g/mol) and produces a fragment ion (*m/z* 140) that undergoes heterolytic cleavage to expel a molecule of ethene and produce another fragment ion (*m/z* 112). The cleavage processes then continue by loss of a CH_2 fragment and produces the dominant fragmentations *m/z* 97, 83, 69 and 67. Both mass spectra have base peaks at *m/z* 55. Ten VOCs were successfully extracted, separated and identified using both the polar and non-polar GC columns as indicated by their retention times and mass spectrum of each compound. Table 4.1 shows the liberated VOCs from each *Salmonella* strain that was grown overnight in TSB at 37 ºC and extracted with a polar SPME fiber and detected using a polar GC column. Table 4.2 shows the physical and chemical properties of the detected VOCs.

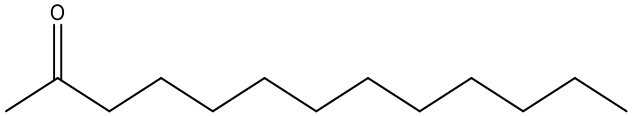
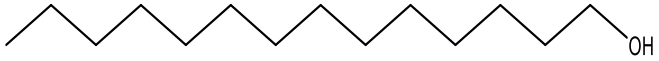
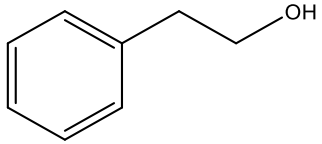
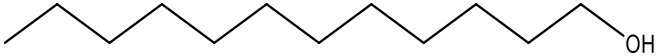
Table 4.1 *Salmonella* VOCs detected by polar GC column and PA SPME fiber after overnight incubation at 37 º C in TSB

***Salmonella* strains**	**VOCs**	
	Common VOCs	**Independent VOC**
S. london	3-Methyl-1-butanol 1-Octanol 1-Decanol 2-Tridecanone 2-Phenyl ethanol Dodecanol 1-Tetradecanol	Ethyl octanoate Ethyl decanoate
S. stanley		
S. oranienburg		Ethyl octanoate Ethyl decanoate
S. othmarschen		Ethyl decanoate
S. gallinarum		2-Heptanone Ethyl octanoate Ethyl decanoate
S. typhimurium		2-Heptanone Ethyl octanoate Ethyl decanoate

Table 4.2 Physical and chemical properties of the detected *Salmonella* VOCs

Compound	Structure	Molar mass (g/mol)	Boiling point	Vapour pressure
2-heptanone		114.2	151° C	2.14 mm Hg (20°C)
3-methyl-1-butanol		88.2	131.1° C	2 mm Hg (20°C)
1-Octanol		130.2	195-196° C	0.14 mm Hg (25°C)
Ethyl octanoate		172.3	206 -208 ° C	0.02 mm Hg (25°C)
Ethyl decanoate		200.3	245 ° C	0.02 mmHg (25 °C)
9-decen-1-ol		156.3	234-238 °C	0.005 mmHg (20 °C)
1-decanol		158.3	232.9 °C	1 mm Hg (70°C)

Continued, Table 4.2. Physical and chemical properties of the detected *Salmonella* VOCs

Compound	Structure	Molar mass (g/mol)	Boiling point	Vapour pressure
2-tridecanone		198.3	133-134°C	0.02 mm Hg (25 °C)
1-tetradecanol		214.4	289 °C	0.75 mm Hg (20 °C)
2-phenyl ethanol		122.2	219–221 °C	1 mm Hg (58°C)
Dodecanol		186.3	259 °C	0.1 mm Hg (20°C)

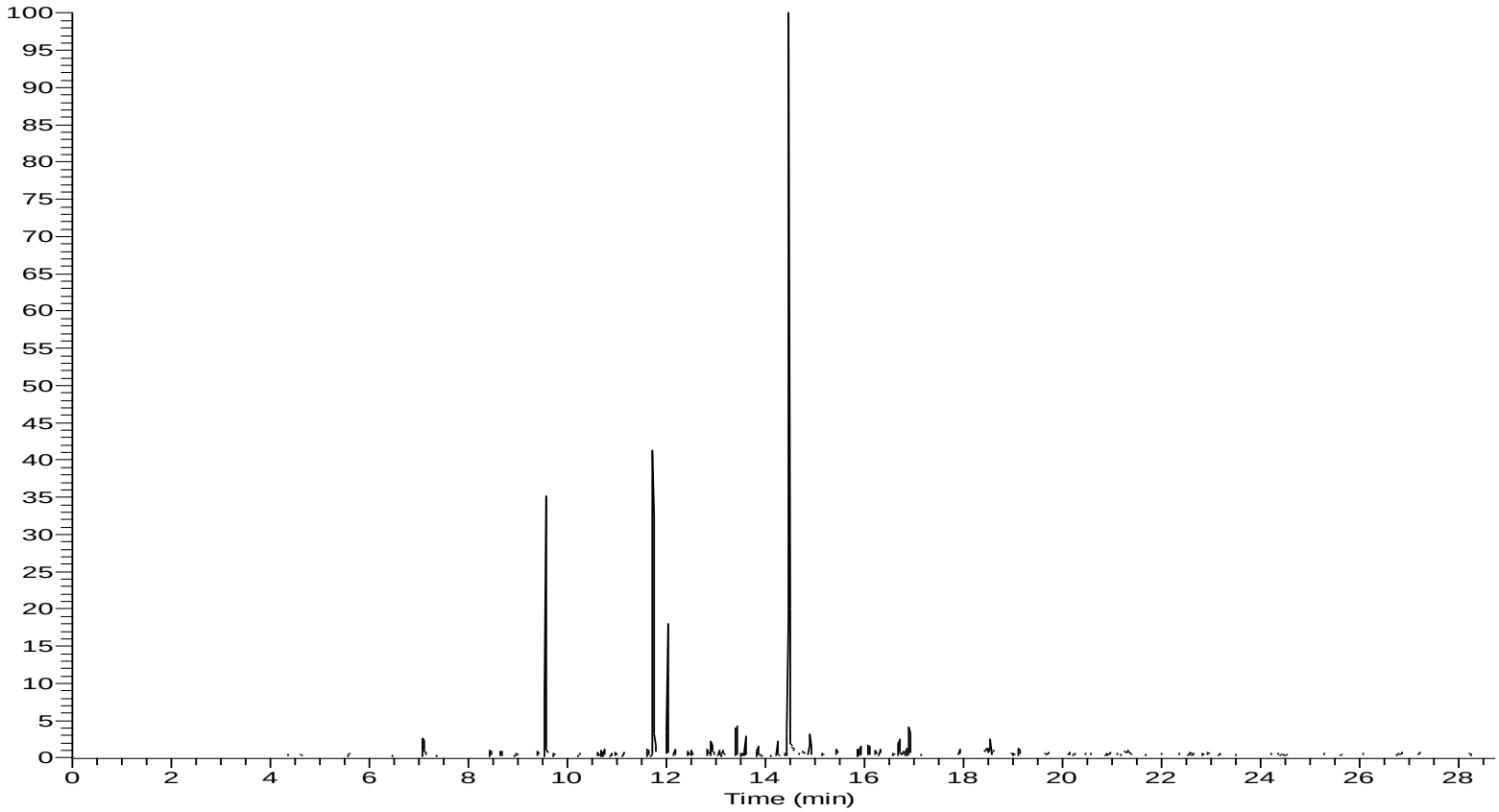


Figure 4.1 Chromatogram showing the VOCs liberated by *S. gallinarum* inoculated in TSB and analyzed with non-polar GC column and non-polar SPME fiber 1-Octanol (t_R 8.66 min), 2-phenyl ethanol (t_R 9.41 min), Ethyl octanoate (t_R 10.69 min), 9-decen-1-ol (t_R 11.63 min), 1-decanol (t_R 11.75 min), Ethyl decanoate (t_R 13.42 min), Dodecanol (t_R 14.42min), 2-Tridecanone (t_R 14.87 min), 1-Tetradecanol (t_R 16.90 min). Other peaks are either unknown compounds from the broth or background noise from the SPME fiber

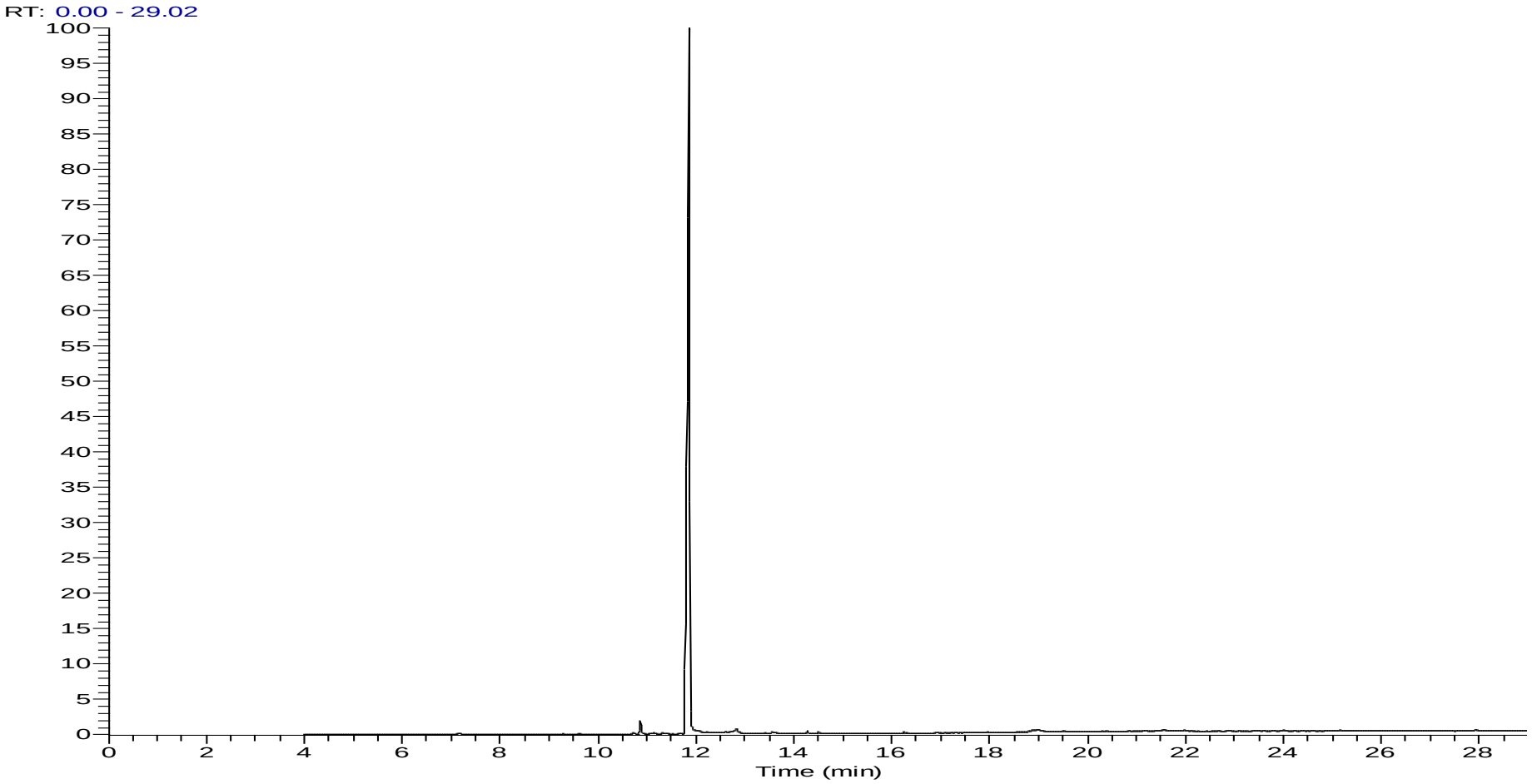


Figure 4.2 Chromatogram of standard 1-decanol (t_R 11.75 min) extracted with PDMS SPME fiber and detected with the non-polar GC column

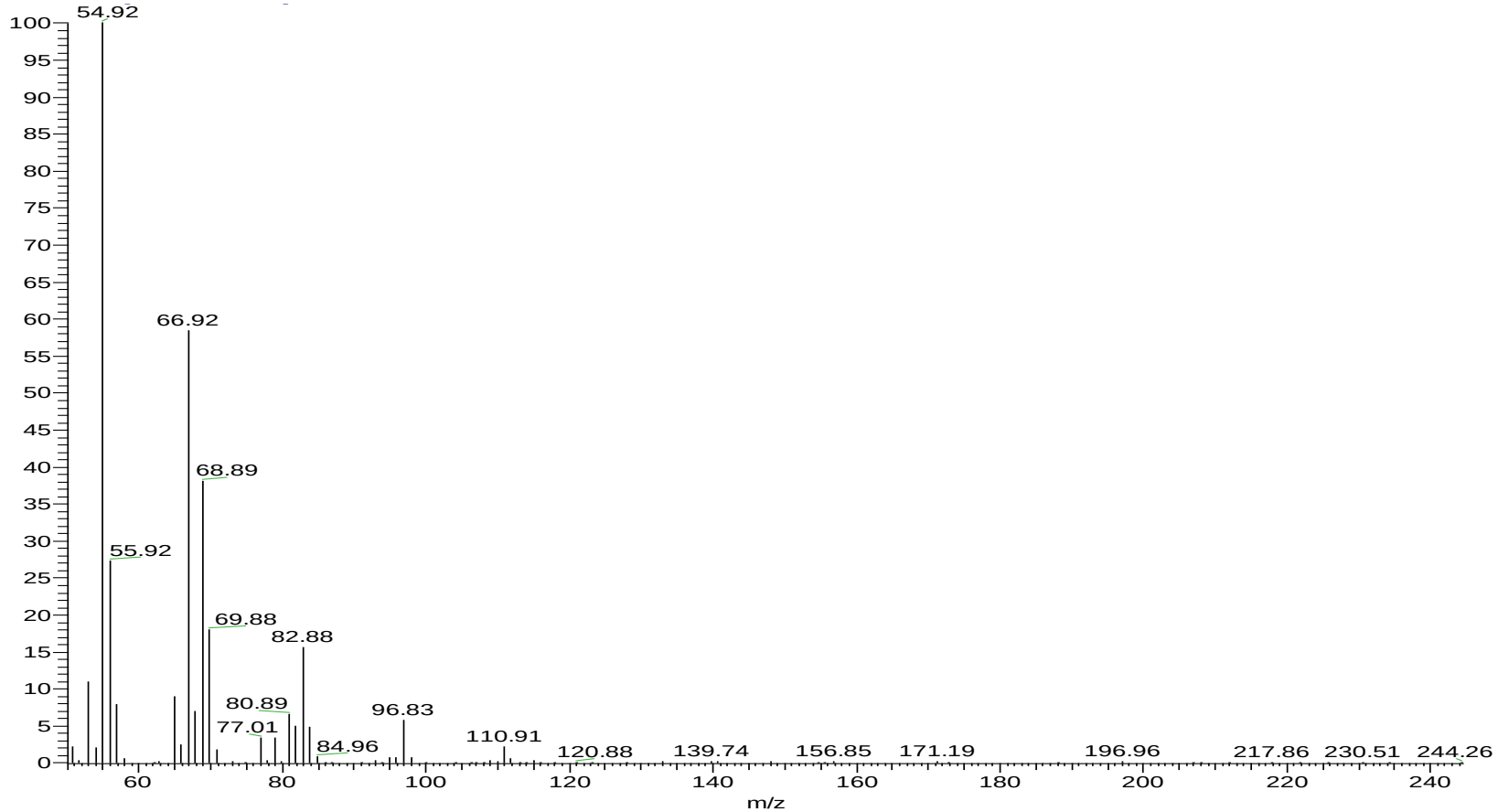


Figure 4.3 The mass spectrum of standard (1 µg/mL) 1-decanol analyzed with non-polar GC column and non-polar SPME fiber

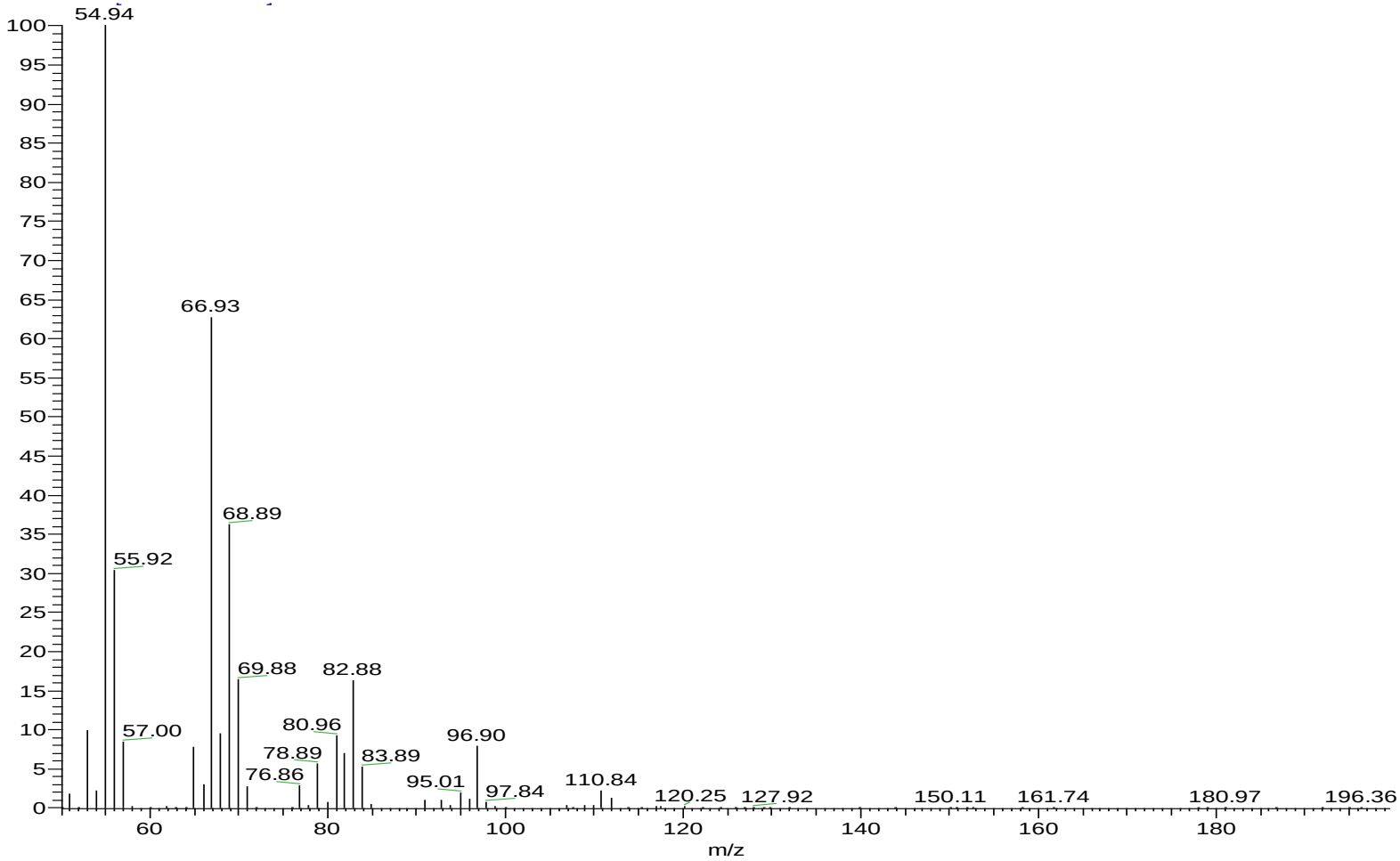


Figure 4.4 The mass spectrum of 1-decanol produced by *S. gallinarum* inoculated in TSB and analyzed with non-polar GC column and non-polar SPME fiber

Salmonella strains generated alcohol compounds as the dominant type of VOCs. In addition, ester and ketone compounds were also detected. This result may be explained by the fact that volatiles are most likely formed by modification of the breakdown products of fatty acid biosynthetic pathways, for example hydrocarbons, aliphatic alcohols, ketones, and other components (Schulz and Dickschatb 2007). The findings of this study are consistent with those of Arnold and Senter (1998) who found 9-decen-1-ol, 1-butanol, 3-methyl-1-butanol, dodecanol, octanol, and 1-tetradecanol were produced by *Salmonella enteritis* after inoculation in tryptic soya broth and were detected after extraction by a non-polar SPME fiber (PDMS).

The composition of the headspace volatiles detected using either a polar or non-polar column was the same with the exception of two compounds. The first compound is 3-methyl-1-butanol (6.8 min) which was isolated in the headspace of all broth types using the 85 µm polyacrylate (PA) SPME fiber and separated using the polar GC column but was not detected with the non- polar GC column and non-polar SPME fiber (PDMS). Senecal *et al*. (2002) detected 3-methyl-1-butanol in the head space of *S. typhimurium* grown on selected agar medium using SPME GC/MS. In a previous study (Bhattacharjee *et al*., 2011) 3-methyl-1-butanol was detected using HS- SPME/GC-MS in spiked beef samples inoculated with *Salmonella typhimurium* (10^4 CFU/ml) in 0.1 % peptone water and was not detected in control samples. In addition, they found 3-methyl-1-butanol had the most significant change in peak area response with increasing *Salmonella* growth. For this reason, they concluded that 3-methyl-1-butanol could serve as a potential indicator of *Salmonella* contamination of food samples. However, evolution of 3-methyl-1-butanol from other bacteria has been previously reported (Arnold and Senter, 1998; Jia *et al*.; 2010 Tait *et al*., 2013).

The second compound was 9-decen-1-ol (t_R = 11.6 min) which was detected using the non-polar GC column with a 100 µm (PDMS) SPME fiber whereas the polar column with an 85 µm polyacrylate (PA) SPME fiber did not isolate the compound. However, a previous study (Tait *et al*., 2013) reported a separation of 9-decen-1-ol from an *E. coli* strain cultured in TSB using the same polar column and the SPME fiber of 50/30 mm divinylbenzene (DVB)–carboxen (CAR)–PDMS.

The analytical data of the detected VOCs on both the polar and non-polar GC column are shown in Tables 4.3 and 4.4, respectively. All VOCs showed linearity over a five-point concentration range with correlation coefficients above 95%. The values for the LOD were determined as the peak area compared to 3 times the signal-to-noise ratio and LOQ were determined as the peak area compared to 10 times the signal-to-noise ratio. The most sensitive VOCs liberated by *Salmonella* were detected by the non-polar GC column and non-polar SPME fiber and they are; ethyl octanoate and ethyl decanoate with the lowest LOD and LOQ as specified in Table 4.4. The detected 2- phenyl ethanol was the least sensitive VOC with the highest LOD and LOQ in all broths used (as can be seen in Table 4.4).

The variation in sensitivities between the two columns used is clear (Table 4.3 and 4.4). The VOCs were detected with higher sensitivity using the polar GC column and polar SPME fiber (PDMS). For example, ethyl octanoate can be detected and quantified in high sensitivity (LOD = 0.06 ng/mL and LOQ = 0.21 ng/mL in RVS) using the polar system, whereas by using the non-polar system it can be detected in RVS with less sensitivity (LOD = 0.24 ng/mL, and LOQ = 0.81 ng/mL in RVS).

Table 4.3 Calibration data for *Salmonella* VOCs using the polar GC column and polar SPME fiber (PA). The linear range is 0.05-1.5 µg/mL (n = 5)

Compound	Retention time (t_R ; min)	Equation (y = mx+c)	R^2	LOD (ng/mL)			LOQ (ng/mL)		
				TSB	BHI	RVS	TSB	BHI	RVS
2-Heptanone	6.5	y =82442 x - 4551.7	0.9808	15.5	20.1	34.6	51.5	66.9	115
3-Methyl-1-butanol	6.8	y = 11877x + 556.06	0.9799	40.1	10.8	17.1	13.4	36.0	57.0
Ethyl octanoate	10.0	y = 2E+07 x - 2E+06	0.9987	0.02	0.06	0.06	0.08	0.20	0.21
1-Octanol	11.5	y = 514650 x - 48812	0.9445	1.10	0.35	0.47	3.63	1.17	1.57
Ethyl decanoate	12.5	y =5.00E+07 x - 2.00E+06	0.9904	0.02	0.02	0.07	0.05	0.05	0.22
1-Decanol	13.9	y = 5.00E+06 x - 387552	0.9983	1.47	1.33	1.05	4.09	4.04	3.51
2-Tridecanone	14.5	y = 3.00E+07 x - 38733	0.9933	0.87	0.44	0.73	2.90	1.46	2.43
2-Phenyl ethanol	15.7	y = 94550 x+ 846.47	0.9708	56.4	14.6	14.3	18.8	48.7	47.6
Dodecanol	16.1	y = 8.00E+06 x + 17420	0.9939	1.90	4.28	4.50	6.32	14.3	15.0
1-Tetradecanol	18.1	y = 4.00E+06 x + 1252.2	0.9999	5.85	8.37	8.45	19.5	27.9	28.2

n = number of points on calibration curve, LOD = limit of detection, LOQ = limit of quantification

Table 4.4 Calibration data for *Salmonella* VOCs using the non- polar GC column and nonpolar SPME fiber (PDMS). The linear range is 0.05-1.5 µg/mL (n = 5)

Compound	Retention time (t_R ; min)	Equation (y = mx+c)	R^2	LOD (ng/mL)			LOQ (ng/mL)		
				TSB	BHI	RVS	TSB	BHI	RVS
2-Heptanone	5.6	y = 81354 x – 1657.9	0.9758	34.0	120	186	113	400	619
1-Octanol	8.6	y = 304608 x- 7115.6	0.9926	9.00	8.00	11.0	30.0	25.0	36.2
2-Phenyl ethanol	9.4	y = 18625 x- 181.56	0.9988	539	257	369	1796	857	1232
Ethyl octanoate	10.6	y = 3.00E+07 x- 994956	0.9682	0.12	0.22	0.24	0.39	0.75	0.81
9-Decen-1-ol	11.6	y = 1.00E+06 x - 51504	0.9811	3.39	26.9	6.76	11.3	89.5	22.5
1-Decanol	11.7	y = 4.00E+06 x + 43444	0.9935	1.22	71.6	1.47	4.06	13.4	4.90
Ethyl decanoate	13.4	y = 8.00E+07 x + 1.00E+06	0.9893	0.17	0.16	0.21	0.56	0.54	0.68
Dodecanol	14.4	y = 5.00E+06 x + 1.00E+06	0.9594	3.44	1.71	1.48	11.5	5.69	4.92
2-Tridecanone	14.7	y = 4.00E+07 x + 1.00E+06	0.9746	0.72	2.48	0.27	2.40	8.25	0.90
1-Tetradecanol	16.9	y = 2.00E+06 x+ 1.33E+05	0.9786	1.37	13.0	6.72	4.55	43.3	22.4

n = number of points on calibration curve, LOD = limit of detection, LOQ = limit of quantification

4.2.1 Differentiating between *Salmonella* strains grown in 3 types of media via generated VOC profiles

Three different types of broths were evaluated using either a polar or non-polar GC column. Table 4.5, 4.6, and 4.7 shows the concentrations of detected VOCs using the polar GC column and a polar SPME fiber while Table 4.8, 4.9 and 4.10 shows the VOC concentrations when using the non-polar GC column and a non-polar SPME fiber. Nine VOCs were separated using the 3 broths in both columns; however, each column additionally was able to separate one compound that the other one did not separate. The polar GC column combined with the polar SPME fiber was able to separate 3-methyl-1-butanol, while the non-polar column combined with the non-polar SPME fiber was able to separate 9-decen-1-ol.

Variation in types of VOCs detected could be due to the variety of culture medium components. The RVS broth is the preferred selective enrichment medium for the isolation of *Salmonellae;* it contains soya peptone (4.5 g/L) as the nitrogen and vitamin source to enhance the growth of *Salmonella* strains (Van Schothorst and Renaud, 1983), as well as malachite green, which is used to inhibit some other bacteria e. g. *Escherichia coli* ATCC 25922 which is used as a negative control. Whereas, BHI broth and TSB broth are both highly nutritious media that support the growth of a wide range of microorganisms and both have a similar composition (Section 3.3). Both BHI and TSB contain glucose (2.0 and 2.5 g/L), and both broths have peptone (15 g/L and 17 g/L, respectively) as a complex amino acid/nitrogen source.

Therefore, it is not surprising that the *Salmonella* VOC profiles detected using both columns with the broth types are comparable. Similar VOCs profiles were detected on both *S. Gallinarum* and *S. Typhimurium* inoculated in TSB and

BHI broths on the polar GC column using the polar SPME fiber. However, in the RVS broth the VOCs liberated by both strains are slightly different. Therefore, these two strains could be differentiated when grown in RVS and not in TSB and BHI broths. However, using the non-polar system, these two strains cannot be distinguished in the 3 broth types, as they liberated similar VOCs. In the same way, with the polar system, *S. Oranienburg* and *S. Othmarschen* cannot be distinguished when grown in RVS and TSB, however they could be differentiated when using BHI, as 2-tridecanone, 2-phenyl ethanol and dodecanol, were liberated by *S. Oranienburg* and not by *S. Othmarschen.*

Due to the difference in the types and the quantities of ingredients of the RVS broth and both the BHI and TSB broths, it is evidenced that the *Salmonella* grown in RVS broth generate very different VOC profiles than those grown in BHI and TSB broths. The variation in concentrations and types of VOCs detected could be due to the growth level of the *Salmonella* strains in the growth media. This is in agreement with earlier observations (Robacker *et al*., 2009) which showed that make-up of the odours (VOCs) depends on the type of media used to culture the bacteria in addition to the inherent metabolic capabilities of the bacteria. Therefore, it could be impossible to identify bacteria based on the quantity of the VOCs liberated.

This is highlighted in Figures 4.5, 4.6 and 4.7, where the mean amount of 3-methyl-1-butanol, and 1-octanol liberated by 6 strains of *Salmonella* ± 1 standard deviation are shown respectively. The observed overlap between the amount of 3-methyl-1-butanol and 1-octanol generated by the 6 strains of *Salmonella* could be attributed to the difficulty in differentiating *Salmonella* strains based on the quantity of the VOCs liberated.

Table 4.5 Volatile profiles for *Salmonella* strains inoculated in Tryptone Soya Broth (TSB) on the polar GC column and polar SPME fiber [Mean VOC concentration (ng/mL) (n = 3)]

Compound	*S. london*	*S. stanley*	*S. oranienburg*	*S. othmarschen*	*S. gallinarum*	S. typhimurium
2-Heptanone	ND	ND	ND	ND	131 ± 9.0	175 ± 18.0
3-Methyl-1-butanol	806 ± 227	1301 ± 196	1452 ± 463	1193 ± 335	2324 ± 257	2084 ± 245
Ethyl octanoate	15.0 ± 0.1	ND	21.1 ± 9.0	ND	17.5 ± 2.0	28.0 ± 21.0
1-Octanol	78.0 ± 0.7	81.0 ± 2.4	12.5 ± 6.0	13.0 ± 4.0	100 ± 3.0	99.9 ± 3.0
Ethyl decanoate	29.7 ± 2.0	ND	63.0 ± 17.0	44.9 ± 0.1	42.0 ± 4.0	82.6 ± 60
1-Decanol	75.0 ± 2.0	79.0 ± 1.0	10.2 ± 14.0	86.4 ± 1.0	10.8 ± 2.0	99.0 ± 13.0
2-Tridecanone	9.0 ± 1.0	0.60 ± 0.20	25.8 ± 2.70	4.70 ± 1.0	8.62 ± 4.0	55.3 ± 7.0
2-Phenyl ethanol	94.8 ± 14.6	109 ± 37.0	171 ± 55.0	334 ± 32.5	134 ± 32.0	101 ± 38.0
Dodecanol	19.3 ± 6.0	24.9 ± 6.0	72.2 ± 20.0	38.0 ± 6.0	51.8 ± 5.0	71.0 ± 58.0
1-Tetradecanol	14.0 ± 0.9	8 .0.± 1.0	65.0 ± 22.0	44.0 ± 1.0	22.8 ± 13.0	65.9 ± 23.0

ND = not detected

Table 4.6 Volatile profiles for *Salmonella* strains inculated in Brain Heart Infusion Broth (BHI) on the polar GC column and polar SPME fiber [Mean VOC concentration (ng/mL) (n = 3)]

Compound	*S. london*	*S. stanley*	*S. oranienburg*	*S. othmarschen*	*S. gallinarum*	S. typhimurium
2-Heptanone	86 ± 25	86 ± 26	ND	ND	ND	ND
3-Methyl-1-butanol	2719 ± 188	4596 ± 165	1328 ± 498	1207 ± 129	1630 ± 128	1207 ± 222
Ethyl octanoate	112 ± 2.0	111 ± 2.0	ND	ND	185 ± 0.10	185 ± 0.03
1-Octanol	142 ± 3.0	139 ± 3.0	113 ± 2.0	117 ± 0.10	149 ± 1.0	152 ± 1.0
Ethyl decanoate	95.8 ± 9.0	87 ± 2.0	ND	ND	ND	ND
1-Decanol	137 ± 9.0	136 ± 5.0	140 ± 3.0	145 ± 3.0	162 ± 5.0	146 ± 2.0
2-Tridecanone	10.2 ± 7.0	6.89 ± 0.8	3.13 ± 0.01	ND	ND	ND
2-Phenyl ethanol	71 ± 7.7	38.0 ± 4.5	173 ± 12.8	ND	247 ± 29.0	281 ± 7.0
Dodecanol	27.4 ± 2.2	30.5 ± 5.0	10.6 ± 4.0	ND	67.5 ± 15.0	44.7 ± 9.0
1-Tetradecanol	7.43 ± 5.0	8.6 ± 3.0	ND	ND	6.66 ± 0.60	14.1 ± 3.70

ND = not detected

Table 4.7 Volatile profiles for *Salmonella* strains inoculated in Rappaport-Vassiliadis Soya Peptone Broth (RVS) on the polar GC column and polar SPME fiber [Mean VOC concentration (ng/mL) (n = 3)]

Compound	*S. london*	*S. stanley*	*S. oranienburg*	*S. othmarschen*	*S. gallinarum*	S. *typhimurium*
2-Heptanone	ND	110 ± 12.0	ND	ND	89 ± 5.0	N D
3-Methyl-1-butanol	337 ± 90.0	773 ± 123	624 ± 287	663 ± 268	247 ± 117	260 ± 198
Ethyl octanoate	ND	ND	ND	ND	ND	ND
1-Octanol	ND	ND	ND	ND	ND	ND
Ethyl decanoate	ND	ND	ND	ND	ND	ND
1-Decanol	ND	ND	ND	ND	ND	ND
2-Tridecanone	2 ± 0.001	2.0 ± 0.04	ND	ND	ND	1.50 ± 0.04
2-Phenyl ethanol	26.0 ± 53.0	21.0 ± 32.0	15.0 ± 37.0	19.0 ±15.0	46.0 ± 17.0	35.0 ± 4.0
Dodecanol	ND	ND	ND	ND	3.50 ± 0.20	ND
1-Tetradecanol	ND	ND	ND	ND	ND	ND

ND = not detected

Table 4.8 Volatile profiles for *Salmonella* strains inoculated in Tryptone Soya Broth (TSB) on the non-polar GC column and non-polar SPME fiber [Mean VOC concentration (ng/mL) (n = 3)]

Compound	*S. london*	*S. stanley*	*S. oranienburg*	*S. othmarschen*	*S. gallinarum*	S. typhimurium
2-Heptanone	ND	ND	ND	ND	ND	ND
1-Octanol	34.6 ± 4.0	37.0 ± 0.10	< LOQ	< LOQ	37.0 ± 1.80	32.0 ± 1.0
2-Phenyl ethanol	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ethyl octanoate	3.0 ± 0.60	3.0 ± 0.04	32.9 ± 0.10	33.0 ± 0.03	37.1 ± 0.02	37.0 ± 0.01
9-Decen-1-ol	ND	ND	ND	ND	39.3 ± 0.70	37.4 ± 1.0
1-Decanol	9.0 ± 2.0	9.30 ± 2.0	12.2 ± 3.0	3.20 ± 0.50	40.5 ± 5.0	9.82 ± 0.40
Ethyl decanoate	3.10 ± 1.70	3.20 ± 3.0	17.2 ± 14.0	ND	16.0 ± 6.0	105 ± 12.0
Dodecanol	206 ± 53.0	262 ± 84.0	398 ± 15.0	38.5 ± 5.50	774 ± 70.0	196 ± 38.0
2-Tridecanone	94.3 ± 0.10	38.1 ± 17.0	120 ± 10.0	ND	14.6 ± 0.01	156 ± 60.0
1-Tetradecanol	7.60 ± 11.9	33.9 ± 5.70	446 ± 49.0	ND	22.2 ± 0.01	7.0 ± 13.6

ND = not detected; < LOQ = an amount lower than quantification limit

Table 4.9 Volatile profiles for *Salmonella* strains cultured in Brain Heart Infusion Broth (BHI) on the non-polar GC column and non-polar SPME fiber [Mean VOC concentration (ng/mL) (n = 3)]

Compound	*S. london*	*S. stanley*	*S. oranienburg*	*S. othmarschen*	*S. gallinarum*	S. typhimurium
2-Heptanone	ND	ND	ND	ND	ND	ND
1-Octanol	38 ± 5.0	42 ± 7.0	41 ± 2.0	47 ± 3.0	44 ± 1.0	45 ± 1.0
2-Phenyl ethanol	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ethyl octanoate	36.9 ± 0.3	36.7 ± 0.2	35.8 ± 0.4	35.8 ± 0.2	ND	ND
9-Decen-1-ol	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
1-Decanol	32.2 ± 12.0	28.0 ± 8.0	15.1 ± 1.6	19.1 ± 1.5	34.1 ± 4.6	15.3 ± 2.2
Ethyl decanoate	NQ	NQ	NQ	NQ	NQ	NQ
Dodecanol	NQ	NQ	NQ	NQ	NQ	NQ
2-Tridecanone	NQ	NQ	NQ	NQ	NQ	NQ
1-Tetradecanol	< LOQ	NQ	NQ	NQ	NQ	NQ

ND = not detected; < LOQ = an amount lower than quantification limit; NQ = detected but at negative value of concentration

Table 4.10 Volatile profiles for *Salmonella* strains cultured in Rappaport-Vassiliadis Soya Peptone Broth (RVS) on the non-polar GC column and non-polar SPME fiber [Mean VOC concentration (ng/mL) (n = 3)]

Compound	*S. london*	*S. stanley*	*S. oranienburg*	*S. othmarschen*	*S. gallinarum*	S. typhimurium
2-Heptanone	ND	ND	ND	ND	ND	ND
1-Octanol	ND	ND	ND	ND	ND	ND
2-Phenyl ethanol	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ethyl octanoate	23.9 ± 0.01	24.1 ± 0.10	20.1 ± 0.30	20.0 ± 0.10	ND	ND
9-Decen-1-ol	ND	ND	ND	ND	ND	ND
1-Decanol	ND	< LOQ	ND	< LOQ	ND	ND
Ethyl decanoate	ND	< LOQ	< LOQ	< LOQ	ND	ND
Dodecanol	< LOQ	ND	< LOQ	ND	< LOQ	< LOQ
2-Tridecanone	< LOQ	< LOQ	< LOQ	ND	ND	< LOQ
1-Tetradecanol	< LOQ	ND	ND	ND	ND	ND

ND = not detected; < LOQ = an amount lower than quantification limit; NQ = detected but at negative value of concentration

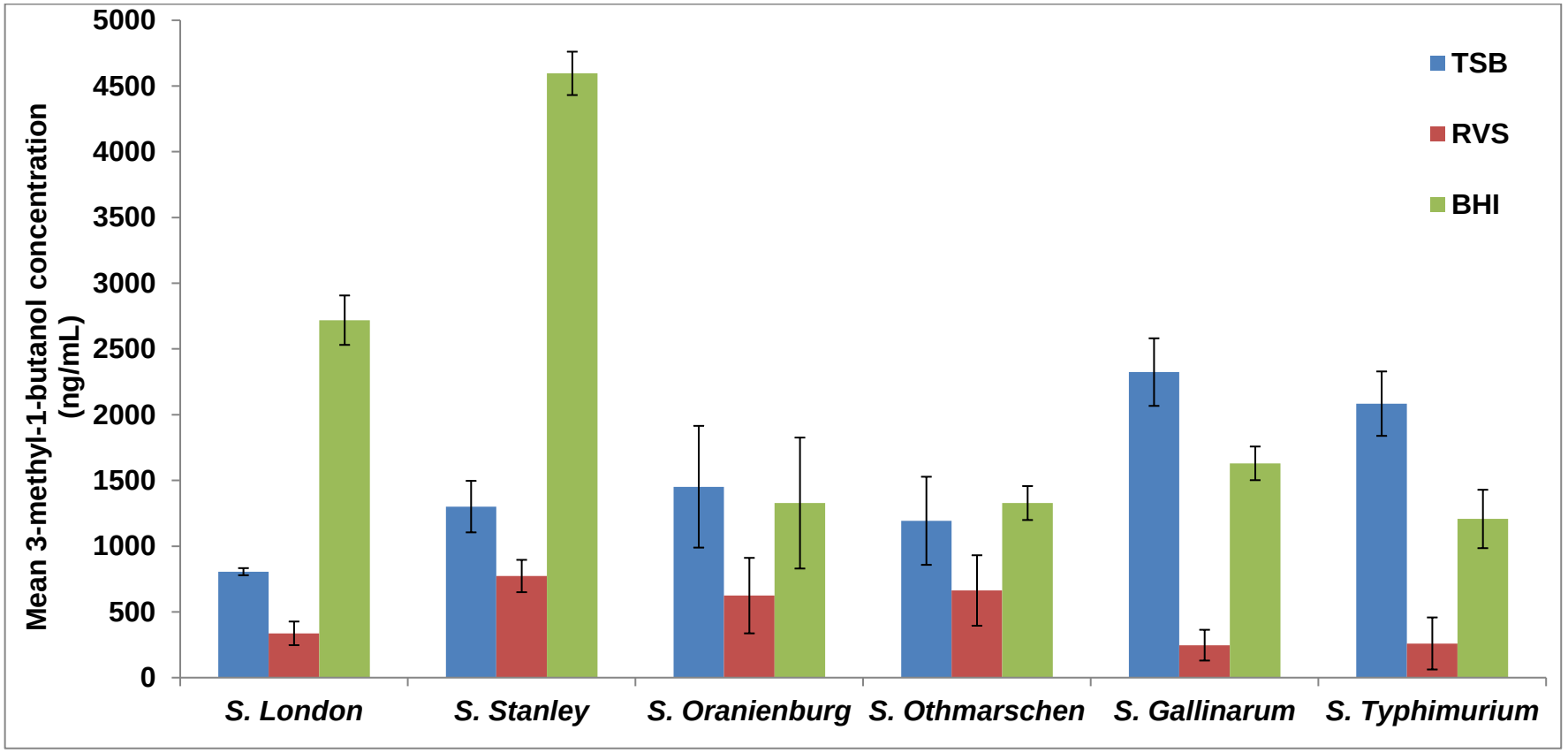


Figure 4.5 Mean of 3-methyl -1-butanol (n = 3) concentration liberated by 6 strains of *Salmonella* inoculated in 3 different broths and detected using a polar GC column and extracted with a polar SPME fiber

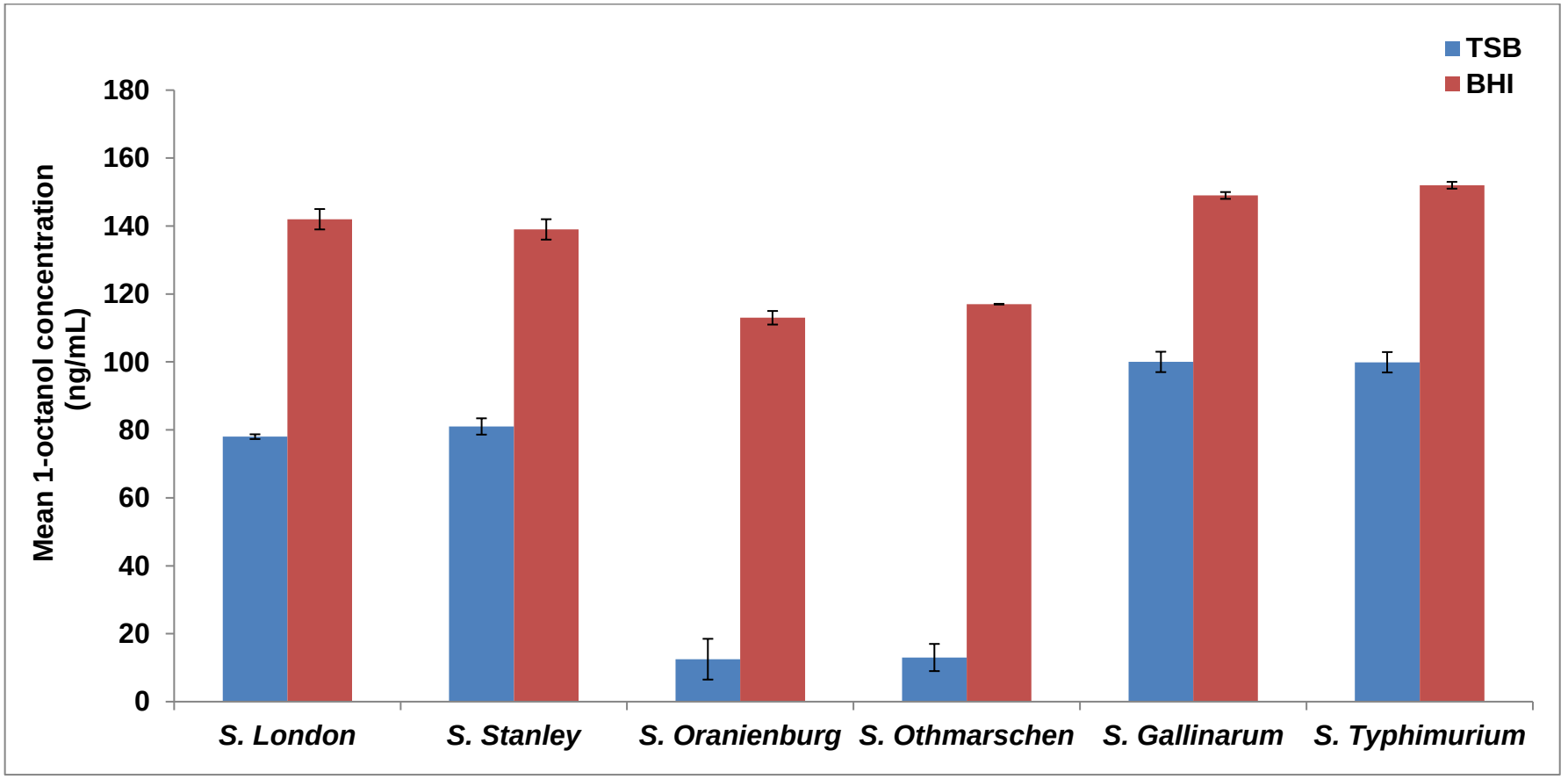


Figure 4.6 Mean of 1-Octanol liberated by 6 strains of *Salmonella* inoculated in 2 different broths and detected using a polar GC column and extracted with a polar SPME fiber

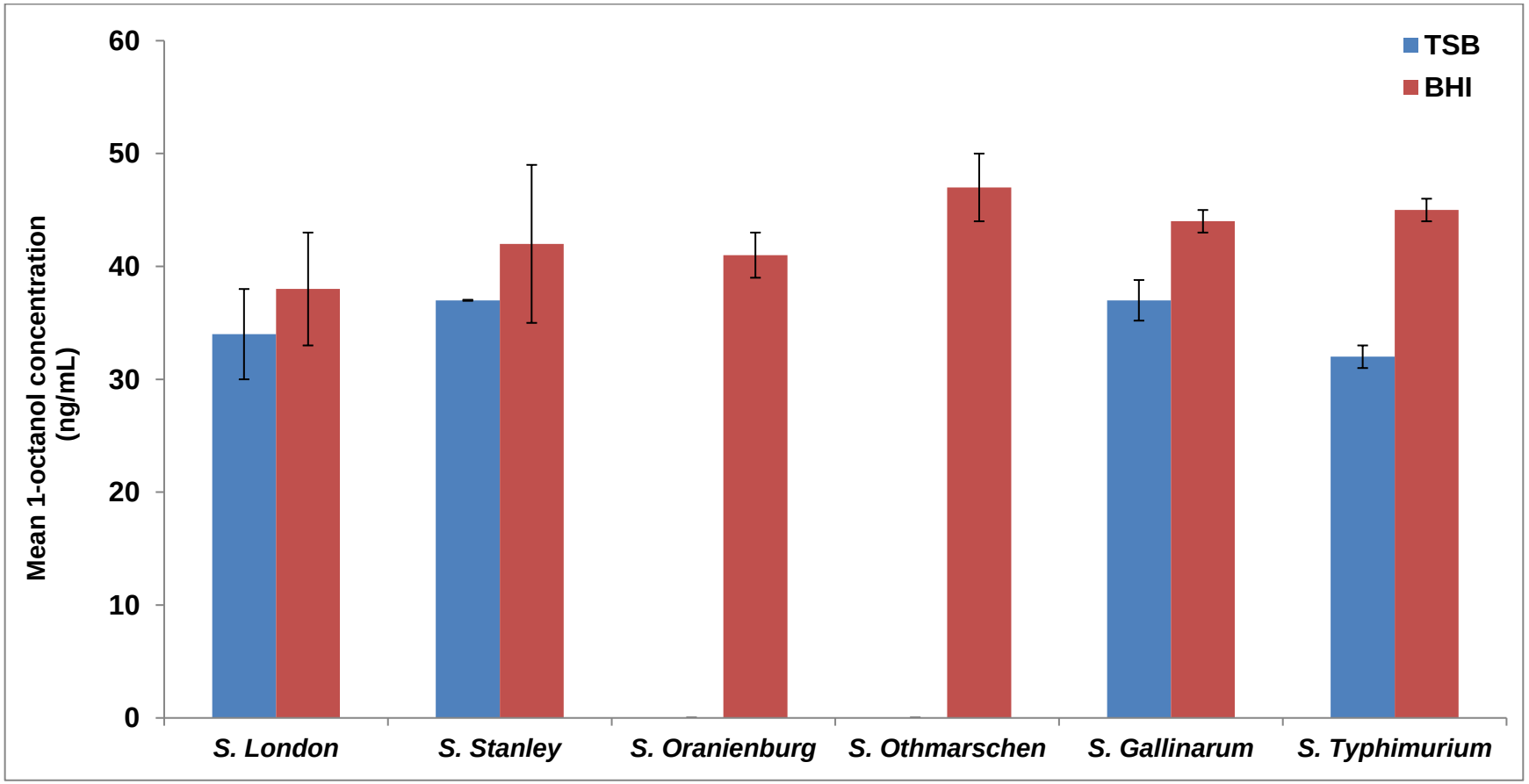


Figure 4.7 Mean of 1-Octanol liberated by 6 strains of *Salmonella* inoculated in 2 different broths and detected using a non-polar GC column and extracted with a non-polar SPME fiber

4.3 Statistical analysis

To establish statistical evidence for the relationship between VOC profile and broth type and also for the purpose of discrimination between the three types of broths used as a growth media the data were subjected to a multivariate analysis method specifically Principal Component Analysis (PCA). The scatter plot of PC1 and PC2 is shown in Figure 4.8. Where the Principal Components 1 and 2 for 6 strains of *Salmonella* inoculated in 3 type of broths and the VOCs were detected using the polar GC column and polar SPME fiber.

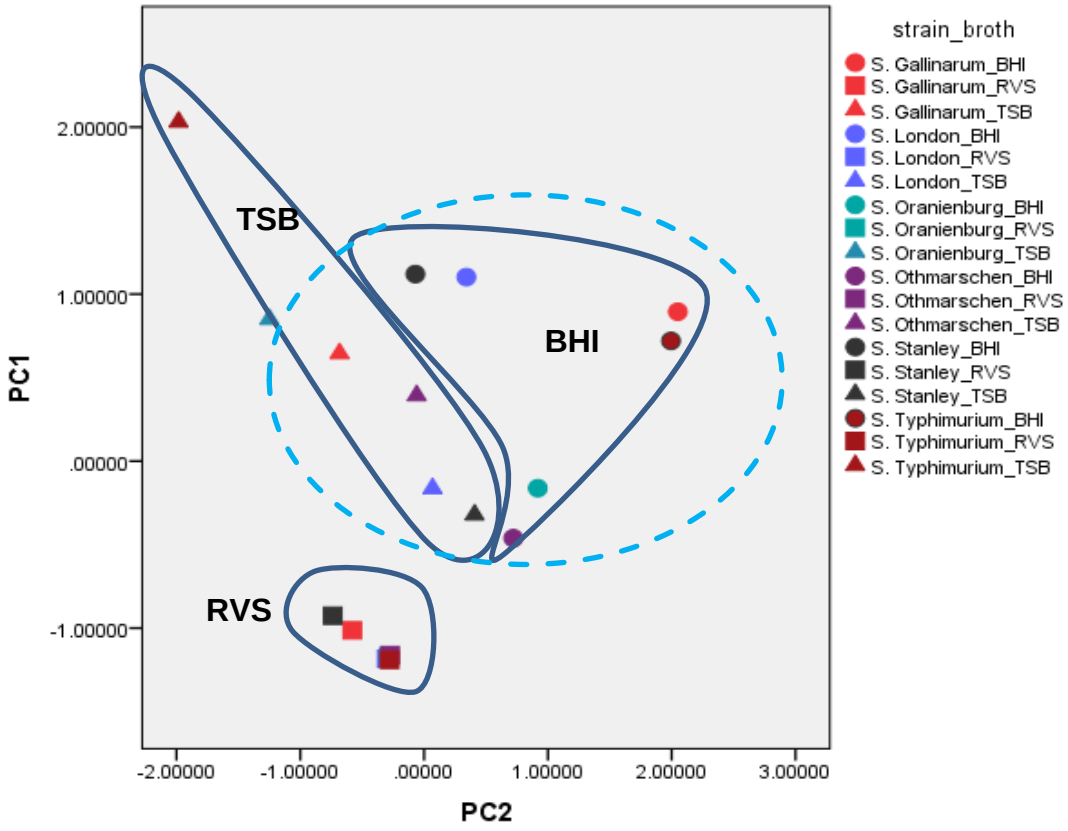


Figure 4.8 PCA scatter plot showing Principal Components 1 and 2 for 6 strains of *Salmonella* in 3 type of broths (VOCs detected using the polar GC column and polar SPME fiber)

While in Figure 4.9 a scatter plot showing Principal Components 1 and 2 for 6 strains of *Salmonella* inoculated in the 3 type of broths and VOCs were detected using the non-polar GC column and non-polar SPME fiber.

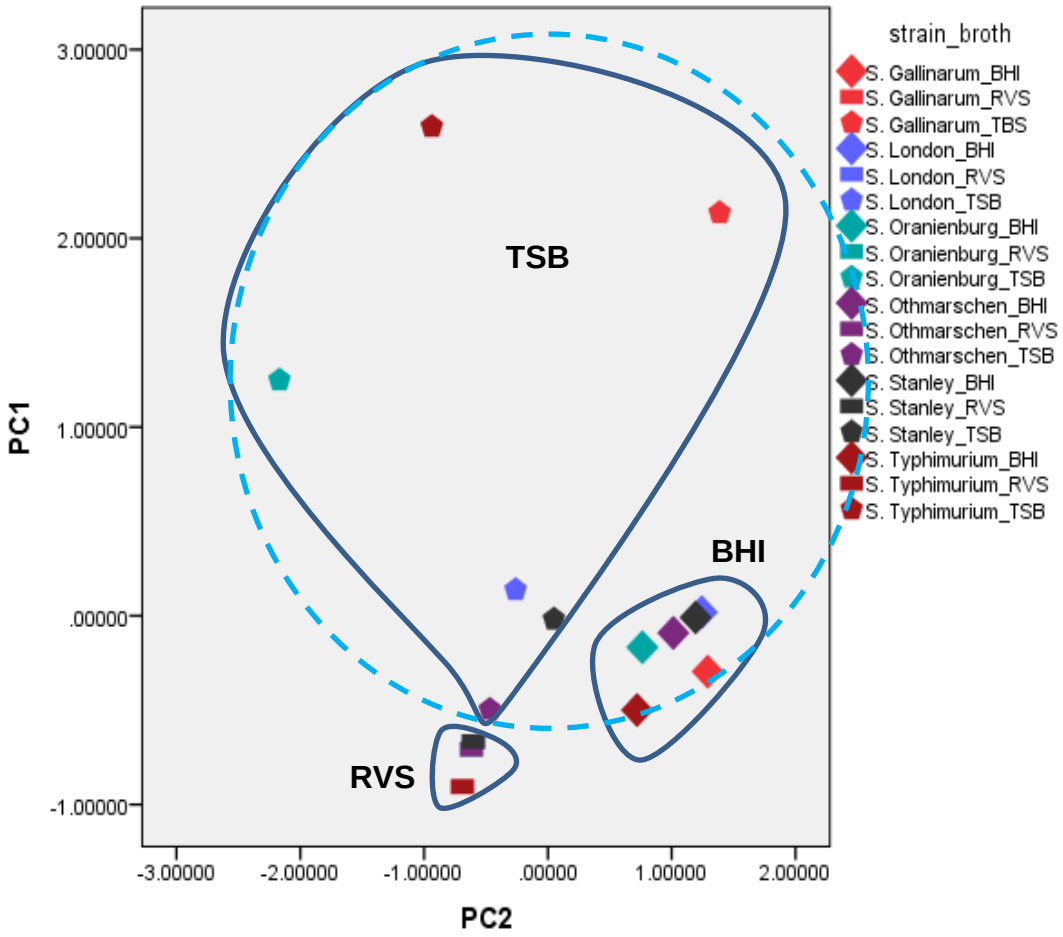


Figure 4.9 PCA scatter plot showing Principal Components 1 and 2 for 6 strains of Salmonella in 3 type of broths (VOCs detected using the non-polar GC column and non-polar SPME fiber)

As can be seen in Figures 4.5 and 4.6 the first two PC`s exhibit a clear separation between the three types of media. However, the two media TSB and BHI are shown to be similar and the RVS broth is clearly distinctive from BHI and TSB broth types on the polar and non-polar GC column as can be seen through the blue dash circle in both Figures.

The scree plots used in principal component analysis to visually assess which components or factors explain most of the variability in the data. A scree plot displays the eigenvalues associated with a component in descending order versus the number of the component. The scree plot (Figure 4.10) shows the

eigenvalue against the VOCs detected using the polar GC and polar SPME fiber. In this scree plot the VOC analysis were conducted on 10 different VOCs. This scree plot shows that 5 of the VOCs (2-heptanone, 3-methyl-1-butanol, ethyl octanoate, 1-octanol, ethyl decanoate) show most of the variability between the 3 types of broths because the line starts to straighten after factor 5 (Table 4.11). 2-Heptanone and 3-methyl-1-butanol explained 51.7% and 25.2% of total variance, respectively. The remaining VOCs explain a very small proportion of the variability and are likely unimportant.

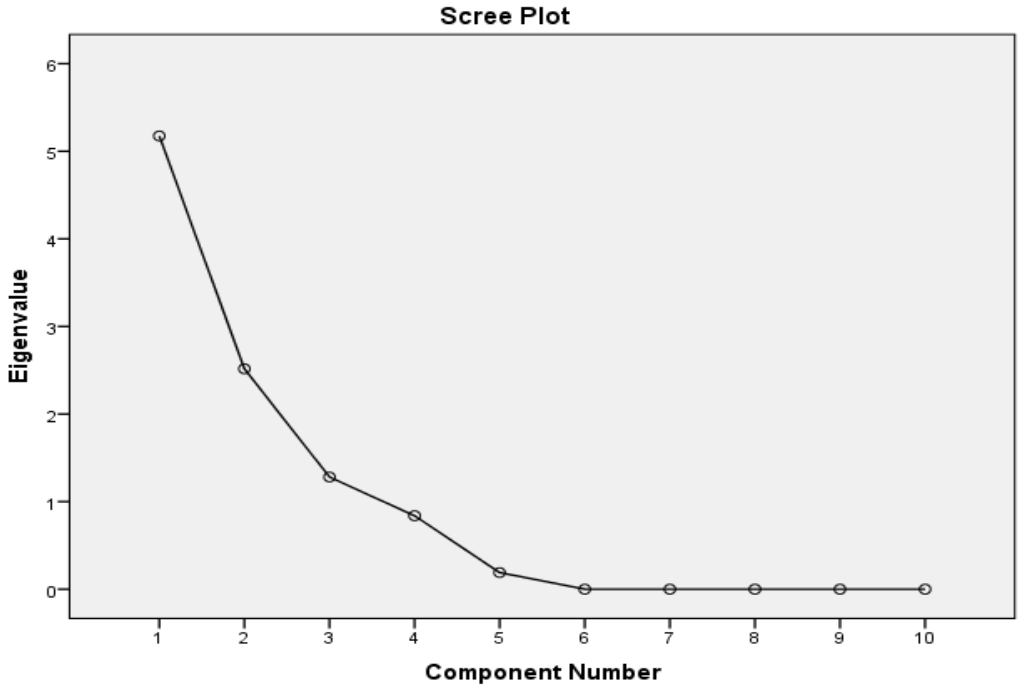


Figure 4.10 The scree plot graphs the eigenvalue against 10 VOCs liberated by 6 strains of *Salmonella* in 3 type of broths (VOCs detected using the polar GC column and polar SPME fiber)

Table 4.11 Total variance explained extraction method: Principal Component Analysis for 6 strains of *Salmonella* in 3 type of broths

Component	Initial Eigenvalues			Extraction Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	5.174	51.744	51.744	5.174	51.744	51.744
2	2.517	25.165	76.909	2.517	25.165	76.909
3	1.281	12.808	89.716			
4	.839	8.389	98.105			
5	.189	1.895	100.000			
6	4.189E-16	4.189E-15	100.000			
7	2.048E-16	2.048E-15	100.000			
8	2.698E-17	2.698E-16	100.000			
9	-1.477E-16	-1.477E-15	100.000			
10	-3.454E-16	-3.454E-15	100.000			

The component: (1) = 2-heptanone, (2) = 3-methyl-1-butanol, (3) = ethyloctanoate, (4) = 1-octanol, (5) = ethyldecanoate, (6) = 1-decanol, (7) = 2-tridecanone, (8) = tetradecanol, (9) = dodecanol, (10) = 2-phenylethanol.

The scree plot in Figure 4.11 displays the eigenvalues associated with the VOCs, liberated by 6 strains of *Salmonella* grown in 3 types of broths detcted by non-polar GC and non-polar SPME fiber, in descending order versus the number of the VOCs. Also, this scree plot shows that 5 of those VOCs (2-heptanone, ethyl octanoate, 1-octanol, ethyl decanoate, and 1-decanol) show most of the variability between the 3 types of broths (Table 4.12). The largest amount of variance in this scree plot was shown by 2-heptanone which was 43.7% of total variance, while ethyl octanoate shows 24.5%. The remaining VOCs show a very small proportion of the variability.

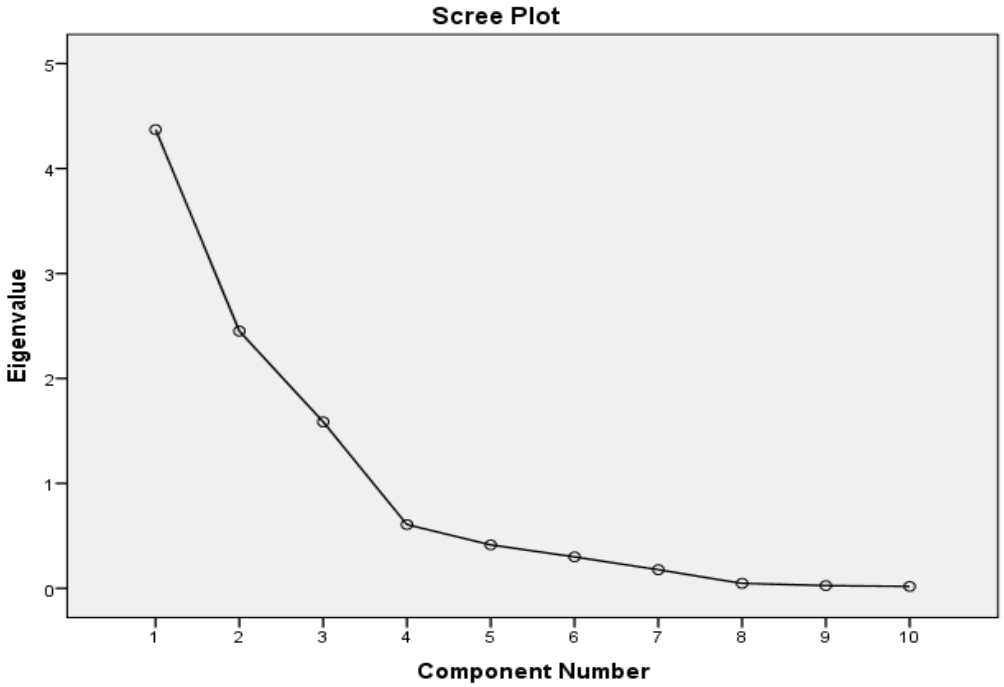


Figure 4.11 The scree plot displays the eigenvalue against 10 VOCs liberated by 6 strains of *Salmonella* in 3 types of broths (VOCs detected using the non-polar GC column and non-polar SPME fiber)

Table 4.12 Total variance explained extraction method: Principal Component Analysis for 6 strains of *Salmonella* in 3 type of broths

	Initial Eigenvalues			Extraction Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	4.370	43.705	43.705	4.370	43.705	43.705
2	2.451	24.510	68.215	2.451	24.510	68.215
3	1.586	15.862	84.077			
4	.608	6.080	90.157			
5	.414	4.142	94.299			
6	.300	3.001	97.300			
7	.178	1.777	99.077			
8	.048	.477	99.554			
9	.026	.263	99.817			
10	.018	.183	100.000			

The component: (1) = 2-heptanone, (2) = ethyloctanoate, (3) = 1-octanol, (4) = ethyldecanoate, (5) = 1-decanol, (6) = 2-tridecanone, (7) = tetradecanol, (8) = dodecanol, (9) = 2-phenylethanol, (10) = 9-decen-1-ol.

4.4 Summary

This study investigated the volatile compounds (gaseous metabolites) produced by *Salmonella* in the headspace of inoculated sterilized broth samples when incubated overnight at 37°C; using HS-SPME in combination with GC-MS. The results of this investigation showed that different types of VOCs were detected. The most important compounds detected were alcohol, ester and ketone compounds.

This study qualitatively explored the profiles of VOCs present in spiked samples. However, it could be impossible to identify *Salmonella* based on the quantity of the VOCs liberated. Culture medium and the polarity of the GC column were found to vary little with detected *Salmonella* VOC profiles. The current study was able to differentiate between some *Salmonella* strains grown in the same media based on their liberated VOCs. However, none of these VOCs could serve as potential indicators of *Salmonella* contamination. The type of the liberated VOC depends on the type of growth media and metabolic capabilities of the strains. Therefore, The VOCs profile of *Salmonella* strains cannot be used as a marker for the presence or absence of *Salmonella* in food samples. And the identification of *Salmonella* by screening for a specific liberated VOCs that act as markers to be employed as a tool for the contaminated food is needed.

More work is recommended for further investigations to identify and quantify the headspace volatiles in spiked food samples. Work needs to be done to identify *Salmonella* on food samples using HS-SPME-GC-MS by recognizing a volatile produced by an enzymatic action on a tailored substrate (a compound that mimics the enzyme`s natural substrate). Attempts to identify bacteria using enzyme-substrates have been reported for more than 20 years (Snyder, 1991a; b, Strachan, 1995). Synthetic enzymatic substrates have been long useful for

both fundamental microbiology and daily analysis of clinical, food and environmental samples (Orenga *et al*., 2009). The next chapter will discuss the use of commercial and synthesised enzyme substrate in growth media as a tool used to aid the identification of *Salmonella* in a sample by detecting the VOCs liberated by *Salmonella* strains during enzymatic activity on this substrate using HS-SPME-GC-MS.

Chapter 5: The use of enzyme substrates in *Salmonella* detection method

5.1 Introduction

As seen in the last chapter the VOCs profile of *Salmonella* strains are not specific to be used as marker for the presence or absence of *Salmonella* in food samples. Therefore, enzyme substrate reactions could improve the specificity of the detection method by monitoring VOCs generated by *Salmonella* during hydrolysis of specific substrates. This chapter discuss the evaluation results of enzyme activities of *Salmonella* (6 strains) in order to develop a detection / identification approach for *Salmonella* in food samples. As the other *Salmonella* detection methods suffered from a lack of specificity this study design is desirable to make the specificity as high as possible. Therefore, it is important to test sufficient enzymes of *Salmonella* to develop unambiguous identification in food samples and not rely on only one enzyme. *Salmonella* enzymes targeted in this study include α-galactosidase, C-8 esterase, pyrrolidonyl peptidase (PYRase) and ornithine and lysine decarboxylase. The hypothesis of the proposed detection method would be that the presence of PYRase activity and the absence of the other three activities would rule out the presence of *Salmonella*. The investigations so far have focussed on detecting *Salmonella* on pure cultures in liquid media without the presence of interfering organisms and without the effect of any food sample matrix. Discussion of the results obtained when incorporating purchased and synthesised enzyme substrates into different inoculated broths will be given. Application to the developed detection approach carried out using the selected enzyme substrates, this is discussed in detail below.

Investigations of some parameters include method sensitivity and effective time required for detection of *Salmonella* is provided. In order to improve the

method specificity an investigation into the enzymatic activities of food related pathogenic bacteria is discussed below.

5.2 Evaluation of *Salmonella* enzymatic activity in pure culture

Salmonella strains used for the enzymatic evaluation are; *S. london, S. oranienburg, S. typhimurium, S. stanley, S. gallinarum* and *S. stanley*. The concentration applied to all of the substrates tested with *Salmonella* strains was 100 µg/mL as this concentration gave a respectable detected signal for the liberated VOCs. The substrates used to evaluate enzymatic activity will now be discussed.

5.2.1 Evaluation of α-galactosidase activity using phenyl α-D-galactopyranoside

Phenyl α-D-galactopyranoside (Figure 3.2) was used to evaluate the α-galactosidase activity in all *Salmonella* strains which is expected to display positive activity (+). This substrate inoculated into the selective RVS broth (Section 3.9.2) and after overnight incubation all the strains demonstrated α-D-galactosidase activity and hydrolysed phenyl α-D-galactopyranoside. The released sugar molecule (galactose) is used by *Salmonella* strains to generate energy for the growth; the liberated phenol was detected in the headspace of the samples using SPME-GC/MS. The detected phenol peak is indicative of the presence of *Salmonella* in the sample, as the positive activity was reported previously in the literature as a marker for most of *Salmonella* strains (Perry and Ford, 2002). The phenol peak was easily identifiable on the chromatogram at a retention time of 15.9 minutes (Figure 5.1a) and its mass spectrum (Figure 5.1 b) was shown to be identical to the standard phenol 25 µg/mL analysed under the same conditions (Figure 5.1c). The amount of phenol liberated by *Salmonella* strains were quantified using external calibration. A calibration graph of phenol

was prepared by spiking standard phenol into 10 mL blank RVS broth. Phenol displayed linearity over a five-point concentration range of 10–100 µg /mL, with correlation coefficients exceeding 0.99. The mean phenol concentrations (µg/mL) liberated by the three replicates with 1 standard deviation per strain was as follows; 7.07 ± 1.46 liberated by *S. London*; 19.11 ± 4.53 liberated by *S. Stanley*; 19.43 ± 6.04 liberated by *S. typhimruim*; 6.10 ± 0. 67 liberated by *S. gallinaruim*; 17.14 ± 4.91 liberated by *S. oranienburg*; 16.24 ±2.53 liberated by *S. othmarchen. S. stanley* liberated the highest amount of phenol as a result of hydrolysing phenyl α-D-galactopyranoside, while *S. gallinaruim* liberated the lowest amount. The production of enzyme begins as soon as the bacteria begin to grow, and the quantities of enzymes produced varies depending on the growth rate. The variation in the detected amount of phenol as indicative of α-galactosidase activity could be due to the different growth rate of *Salmonella* strains.

5.2.2 Evaluation of pyrrolidonyl peptidase (PYRase) activity using L-pyrrollidonyl fluoroanilide

Pyrrolidonyl peptidase (PYRase) activity in 6 strains of *Salmonella* was tested using the synthesised enzyme substrate L-pyrrollidonyl fluoroaniline (Figure 3.1) as well as Section 3.9.2. Hydrolysis of the substrate releases 3-fluoroanaline which could be detected in the headspace of samples containg (positive) PYRase activity organisms. 3-Fluoroanaline was not detected by HS-SPME GC/MS in the 6 strains of *Salmonella* tested. This result clearly indicates the PYRase-negative activity of the 6 *Salmonella* strains tested; this was in accordance with expected results. These findings were well known and have been reported as a distinctive test for *Salmonella* from other bacteria in food samples (Bennett *et al*., 1999; Wenke, 2009).

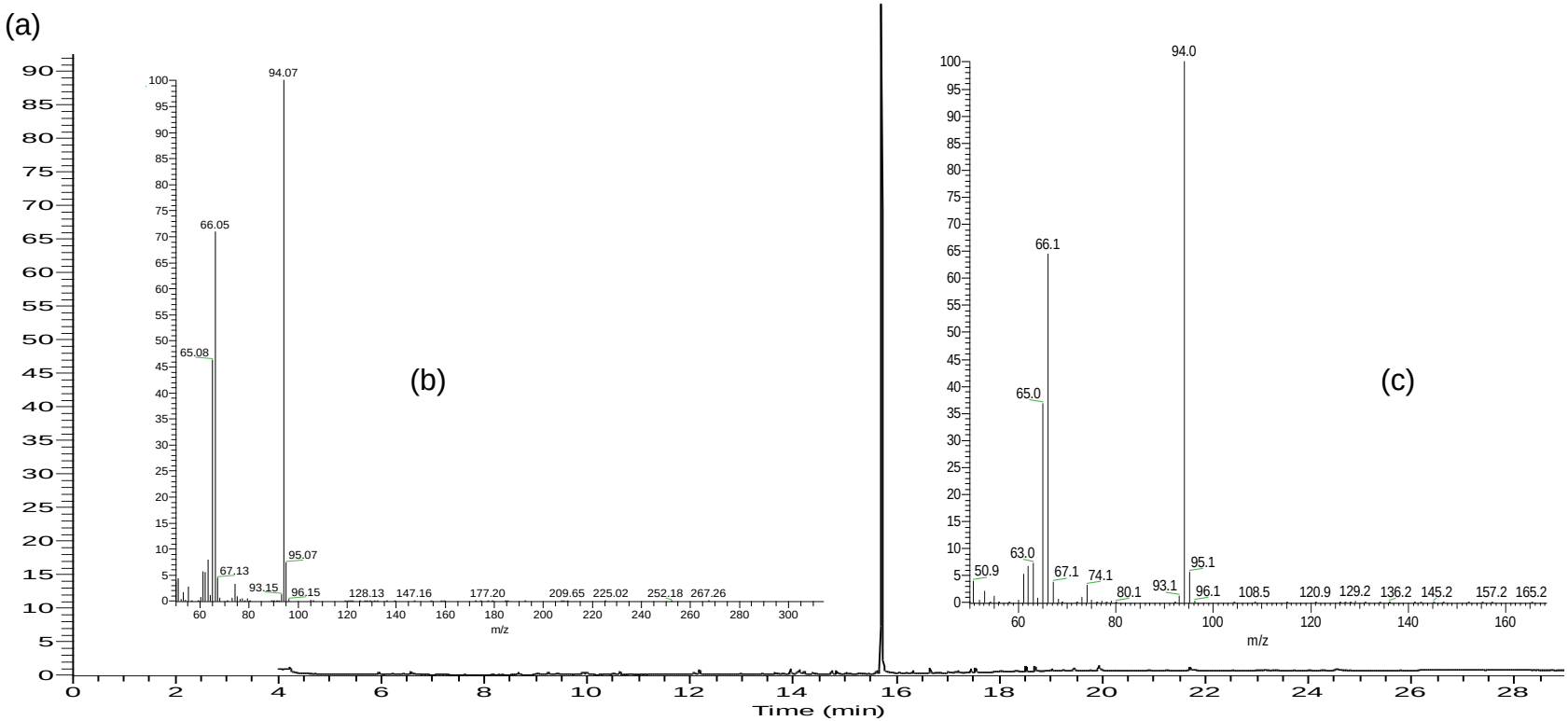


Figure 5.1 (a) is the phenol peak (t_R = 15.9 mins) liberated by *S. stanley* with 100 µg/mL

phenyl α-D-galactopyranoside using a polar GC column and a polar SPME fiber

minor other peaks are unknown compounds evolved from the broth or background compounds from the SPME fibre; (b) is the mass spectrum of phenol generated by *S. stanley* through α-galactosidase activity inoculated in RVS, and (c) is the mass spectrum of standard phenol

For a realisable result, the hydrolysis ability of the synthesised enzyme substrate L-pyrrollidonyl fluoroanilide was tested with bacteria that are PYRase positive, such as *Pseudomonas* (Mulczyk and Szewczuk, 1970). The substrate L-pyrrollidonyl fluoroanilide after inoculation with 1-1.5 x 10^6 CFU *Pseudomonas aeruginosa* (NCTC 10662) detected the VOC 3-fluoroanaline at a t_R of 14.9 mins in the headspace of the samples using SPME GC/MS (Figure 5.2). The chromatogram and mass spectrum of the detected 3-fluoroanaline (Figure 5.2) was identical to the chromatogram and mass spectrum of a 10 µg/mL standard of 3-fluoroanaline (Figure 5.3). These results are obviously illustrating the reliability of the use of L-pyrrollidonyl fluoroanilide as a screening test for PYRase activity in the developed detection method.

5.2.3 Evaluation of C-8 esterase activity using commercial and synthesised esterase substrates

Esterases are found in all living organisms (Manafi *et al*., 1991), and detecting these enzymes in *Salmonella* is well known as an excellent diagnostic marker for the discrimination of *Salmonella* (+) from most other species of Enterobacteriaceae (-) (Aguirre *et al*., 1990).

Commercial and synthesised C-8 esterase substrates were specifically designed and synthesized (Section 3.10) and incorporated into *Salmonella* selective RVS broth to find the suitability and the activity of these different compounds as substrates for detecting *Salmonella* in food samples. More experimental information can be found in Section 3.9.2.3.

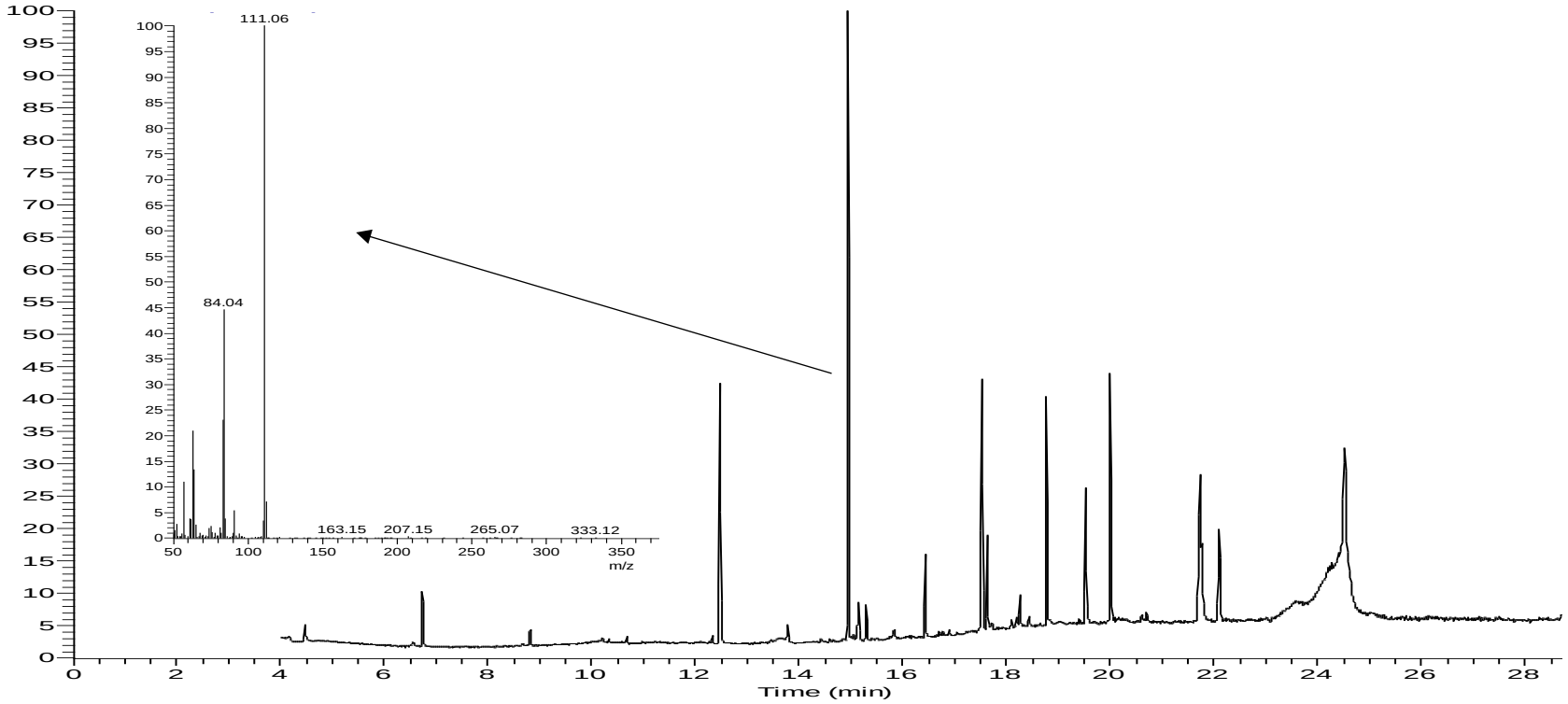


Figure 5.2 Chromatogram and mass spectrum of 3-fluoroaniline (t_R14.9 minutes) liberated by *Pseudomonas aeruginosa* inoculated in TSB and detected with polar GC column and polar SPME fiber

Other peaks are unknown compounds liberated as naturally occurring VOCs by *Pseudomonas aeruginosa* or evolved from the broth or background compounds from the SPME fibre

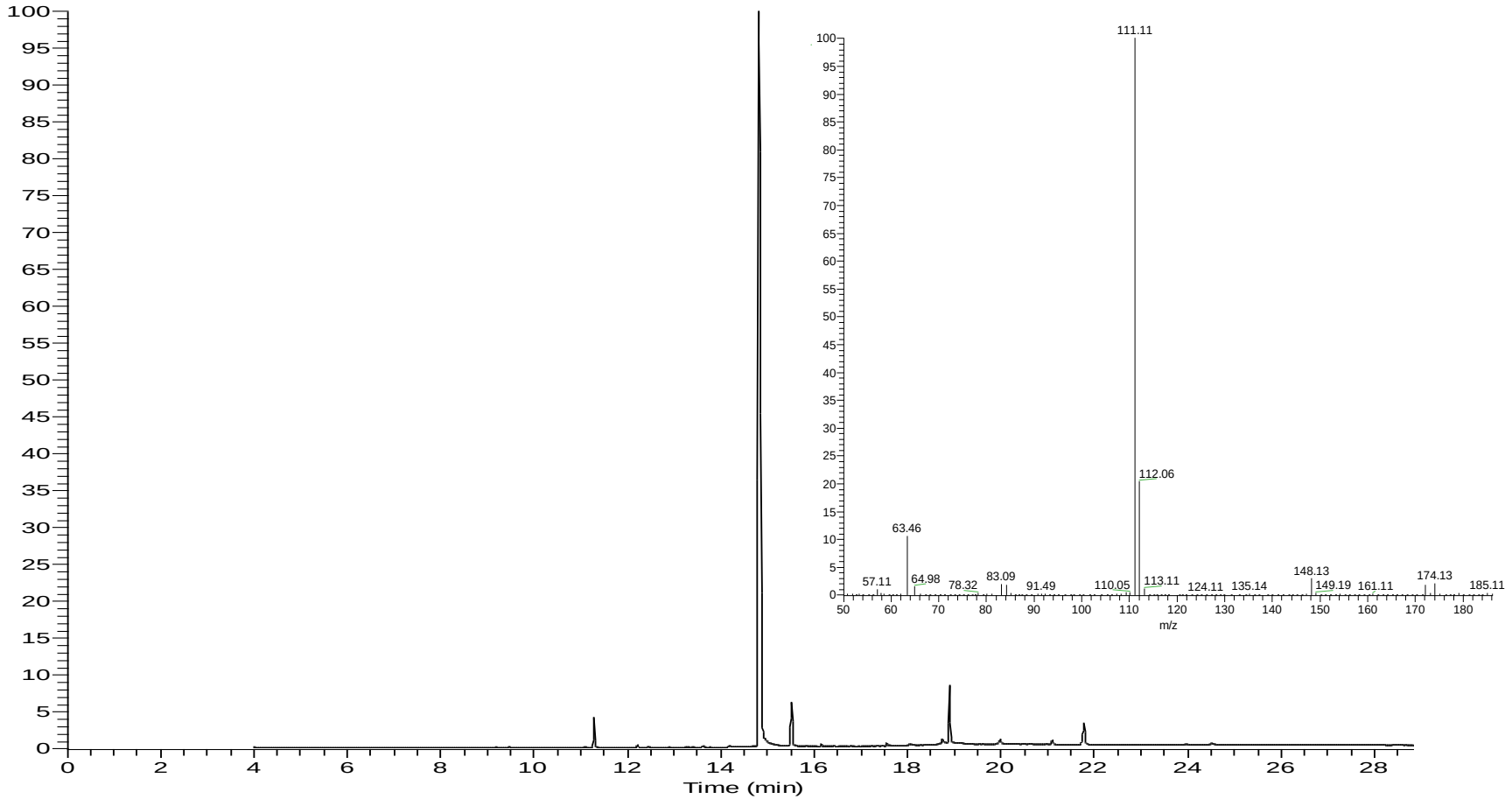


Figure 5.3 Chromatogram and mass spectrum of 10 µg/mL 3-fluoroaniline (t_R 14.82 minutes)

Minor other peaks are unknown compounds evolved from the broth or background compounds from the SPME fibre detected with the polar GC column and polar SPME fiber

As most of the ester compounds are known to hydrolyse in aqueous phase the stability of the C-8 enzymatic substrate in the culture media (broth) is of concern in this study. Therefore, a method to stabilise the C-8 esterase substrates in aqueous solution was needed. Tween 20 (polyoxyethylene (20) sorbitan monolaurate) is a non-ionic detergent widely used in biochemical applications due to its stability and relative nontoxicity. It is highly hydrophilic and acts as a dispersant and solubiliser agent. It has been used as an emulsifying agent for the preparation of stable oil-in-water emulsions. The effect of Tween 20 as well as other detergents have been investigated on the stability of esterase substrates and a 3 g/L of Tween 20 were chosen to use to investigate the stability of the esterase substrate (Nawani *et al*., 1998, Zhang *et al*., 2009).

The results obtained from this study are summarised in Table 5.1. Table 5.1 shows the stability, the *Salmonella* activities and the retention time of the studied substrates and their generated VOCs. In this investigation, commercial isobutyl octanoate (Figure 3.1) was shown to be stable in broth, therefore, testing the esterase activity of *Salmonella* strains using this substrate was carried out. It was concluded from this experiment that *Salmonella* strains are not capable of hydrolysing the substrate isobutyl octanoate as an isobutanol peak was not detected in all *Salmonella* strain samples. It is possible that the factors responsible for the lack of degradation of isobutyl octanoate is caused by hydrolysis due to sample conditions or because *Salmonella* esterase has no active *sites* that can accommodate isobutyl octanoate.

Commercial p-methyl phenyl octanoate, synthesized phenoxy methyl octanoate and synthesized 2,2,2-trifluoroethyl octanoate (Figure 3.1) were all hydrolysed in broth. The peaks of liberated 4-methyl phenol, phenol and 2,2,2-trifluoroethanol were detected in blanks.

In addition, the signal of the detected VOCs did not increase in the *Salmonella* samples indicating that these substrates were fully hydrolysed in the broth. Stabilization of substrates were then investigated by addition of Tween 20 and Tris buffer. The final pH of the broth sample was adjusted to 6.5 using Tris buffer (pH 7, 2 M) as this pH provides optimal conditions for bacteria growth and for substrate solubility as well as for enzyme substrates to react with the enzymes (Todar, 2013). A 3 g/L solution of Tween 20 was added to the samples in the volume range 50% - 300% higher than the volume of NMP in the tested sample. The ratio of Tween 20 to NMP is important as the volume required to solubilize the substrate, and for it to remain in the broth. Also the minimal amount of these should be used so that there is no toxicity towards bacteria. The p-methyl phenyl octanoate, synthesized phenoxy methyl octanoate and synthesized 2,2,2-trifluoroethyl octanoate showed no improvement in their stability in the broth. These results indicate that these substrates are unsuitable to be used in the proposed method.

Evaluation of hexyl octanoate was carried out to predict the ability of *Salmonella* esterases to hydrolyse this type of compound. Hexyl octanoate was found to be more stable in aqueous solution (broth) pH 6.5 when using a 200% higher volume of Tween 20. Therefore, a 100 µg/mL hexyl octanoate was inoculated in *Salmonella* samples. After overnight incubation, *Salmonella* strains hydrolyse this substrate and the hexanol peak was detected. To our knowledge this finding has not been reported in the literature. However, as the liberated VOC is hexanol which is expected to be occur in a natural microbiological system, hexyl octanoate is not a suitable substrate to be used in this detection method. Therefore, 6-chlorohexyl octanoate was synthesized (Section 3.10) to replace the liberated hexanol with chlorohexanol which is unlikely to occur naturally.

6-Chlorohexyl octanoate was stable in the broth so addition of Tween 20 and adjusting the pH was not required. The 6 strains of *Salmonella* hydrolysed 6-chlorohexyl octanoate and liberated 6-chloro-1-hexanol at a retention time of 14.5 minutes. This result was confirmed by detecting the standard 6-chloro-1-hexanol at the same retention time and by mass spectrum identification (Figure 5.4).

A simplification of the *Salmonella* detection method to be easy for use in food applications is planned by detecting the liberated VOC colorimetrically. Unfortunately, the 6-chloro-1-hexanol was not detectable in the headspace colorimetrically. Consequently, alternative C-8 esterase substrates that could be detected colorimetrically were intended to be synthesised and investigated. The 5 phenolic esterase substrates (2,6 dimethyl phenyl octanoate 2-methyl phenyl octanoate, 2-chloro-4-methylphenyl octanoate, 2-nitrophenyl octanoate and 2-chlorophenyl octanoate) were synthesised successfully (Section 3.10). These phenolic substrates were found to be stable in broth so no stabilization reagents were needed. *Salmonella* strains were able to hydrolyse the phenolic C-8 esterase substrates and generated their respective VOCs. These VOCs were detected in the headspace of the samples by GC/MS after overnight incubation at 37 °C. Table 5.1 provides the results obtained.

The results obtained using 2-nitrophenyl octanoate are presented as an example of the phenolic substrates studied. 2-Nitrophenyl octanoate (100 µg/mL) was hydrolysed in broth by all the 6 *Salmonella* strains tested. The 2-nitrophenol peak was easily identifiable on the chromatogram at a retention time of 13.9 minutes (Figure 5.5a) and its mass spectrum was shown to be identical to the standard 2-nitrophenol 10 µg/mL analysed under the same conditions (Figure 5.5b). The amount of 2-nitrophenol liberated by *Salmonella* strains was quantified using external calibration.

Table 5.1 *Salmonella* C-8 esterase substrates and their stability and *Salmonella* strains activities in broth and the retention time of the substrates and their generated VOCs

Substrate	Stability	activity	t_R (min)	VOC	*VOC* t_R (min)
p-Methyl phenyl octanoate	hydrolyse	ND	18	4-methylphenol	16.8
Phenoxy methyl octanoate	hydrolyse	ND	19	phenol	16.0
2,2,2-Trifluoroethyl octanoate	hydrolyse	ND	8.1	2,2,2-trifluoroethanol	5.9
Isobutyl octanoate	stable	ND	11	Isobutanol	4.5
1-Hexyl octanoate	Stable with Tween 20	D	13.9	hexanol	8.3
6-Chloro-1-hexyl octanoate	stable	D	19.1	6-chloro-1-hexanol	14.5
2,6 Dimethyl phenyl octanoate	stable	D	18.6	2,6 dimethyl phenol	14.7
2-Methyl phenyl octanoate	stable	D	18.1	2-methylphenol	15.7
2-Chloro-4-methylphenyl octanoate	stable	D	20.8	2-chloro-4-methylphenol	15.1
2-Nitrophenyl octanoate	stable	D	18.1	2-nitrophenol	13.8
2-Chlorophenyl octanoate	stable	D	19.4	2-chlorophenol	14.1

D = detected, ND = not detect

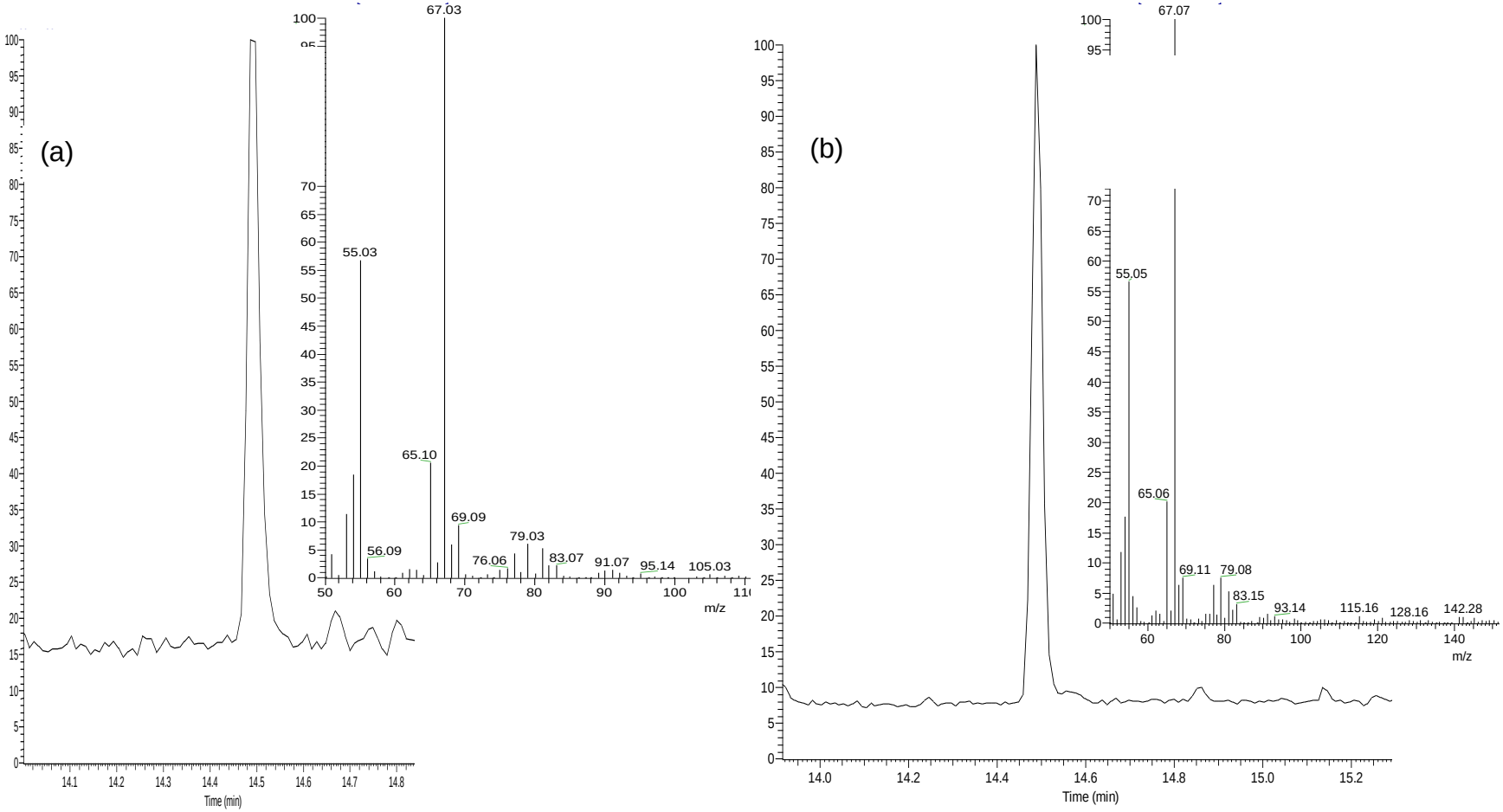


Figure 5.4 Chromatogram and mass spectrum (a); of 6-chloro-1-hexanol (t_R 14.5 min) liberated by *S. london* inoculated in RVS, and (b) standard 10 µg/mL 1-chlorohxanol analysed with polar GC column and polar SPME fiber

A calibration graph for 2-nitrophenol was prepared by spiking a standard 2-nitrophenol into 10 mL blank RVS broth. 2-Nitrophenol displayed linearity over a five-point concentration range of 1–50 µg /mL, with a correlation coefficient exceeding 0.99. The mean 2-nitrophenol concentrations (µg/mL) liberated by the three replicates with 1 standard deviation per strain is as follows; 2.02 ± 0.51 liberated by *S. London*; 2.52 ± 0.33 liberated by *S. Stanley*; 0.70 ± 0.35 liberated by *S. typhimruim*; 3.01 ± 0. 79 liberated by *S. gallinaruim*; 0.60 ± 0.21 liberated by *S. oranienburg*; 2.24 ± 0.97 liberated by *S. othmarchen*. The highest quantity of 2-nitrophenol produced by *S. gallinaruim* however this strain produced the lowest amount of phenol as α-galactosidase activity, while *S.stanley* produced the highest amount of phenol but the second highest amount of the 2-nitrophenol. In addition, 2-nitrophenol can be easily detected colorimetrically in the headspace (Tait *et al*., 2015). Therefore, 2-nitrophenyl octanoate was a most effective substrate for the detection of *Salmonella*. *S. stanley* was selected to investigate the *Salmonella* strains as control samples when testing the food samples.

The results obtained using the phenolic esterase substrates end up with an assay working for *Salmonella* C-8 esterase. Thus, applying C-8 esterase activity as a marker for *Salmonella* detection and identification in food sample is potential.

5.2.4 Detection of decarboxylases activities

Salmonella will utilise the amino acids lysine and ornithine as a source of carbon and energy for growth when the enzyme ornithine decarboxylase catalyses the decarboxylation of ornithine to form putrescine while the enzyme lysine decarboxylase catalyses the decarboxylation of lysine to form cadaverine with liberation of carbon dioxide (Scheme 3.1). The analysis of putrescine and

cadaverine released during *Salmonella* activity needs to be derivatized as part of their sample preparation for GC analysis. They both contain functional groups (-NH) that could form intermolecular hydrogen bonds (Zaikin and Halket, 2003) which affects their volatility and thermal stability; also, the formation of intermolecular hydrogen bonds could interact with the column packing material (Sobolevsky *et al*., 2003). Derivatization by acylation using the reagent trifluoroacetylacetone (TFAA) (Figure 3.3) was used. This reagent was chosen because of its availability and the presence of the trifluoromethyl group which is reported to enhance the volatility of derivatized molecules (Uden, 1984; Khuhawar *et al*., 1999). The next Section will discuss the results obtained from the reaction of putrescine and cadaverine with TFAA in organic solvents.

5.2.4.1 Evaluation of organic phase derivatization

The objective was to investigate the derivatization reaction and determine the retention time and the mass spectra of putrescine and cadaverine derivatives. The experimental details can be found in Section 3.9.2.4.1. The trifluoroacetylacetone (TFAA) reacts with standards of the diamines, cadaverine and putrescine in a 2:1 molar ratio to form H_2TFAA_2PUT and H_2TFAA_2CAD (Scheme 5.1) as reported previously (Khuhawar, 2000; Khuhawar *et al*., 1999). Both compounds were well separated using GC/MS at retention times of 22.9 and 25.5 minutes for putrescine and cadaverene, respectively (Figure 5.7 a, b and c). It is important to note that the molecular ions [M+] for putrescine and cadaverene derivatives in positive ion electron ionization (+EI) mass spectra were m/z 414 and m/z 428, respectively (Figure 5.7). And those corresponding to $[M+54]H^+$ where the principle molecular species (calculated ones) for putrescine derivative should be shown at m/z 361, while the $[M+H]^+$ for cadaverine is m/z 375.

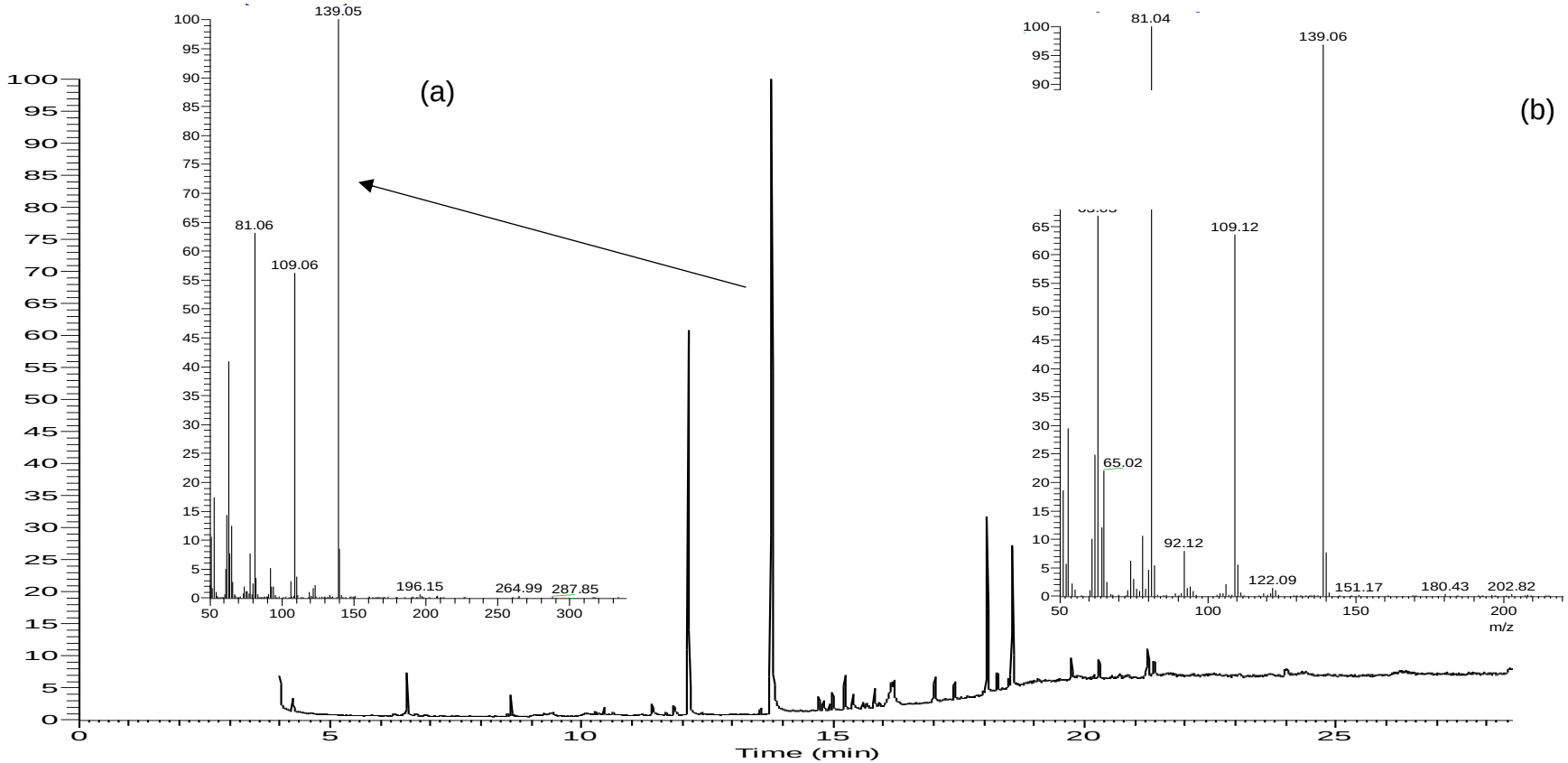


Figure 5.5 (a) chromatogram and the mass spectrum of 2-nitrophenol t_R = 13.8 min liberated by *S. stanley* using HS-SPME GC/MS

t_R = 18.6 min is 2-nitrophenyl octanoate, the solvent NMP, t_R = 12.12 min, other peaks are back ground noise from the SPME fiber and the broth; (b) mass spectrum of standard 2-nitrophenol 10 µg/mL

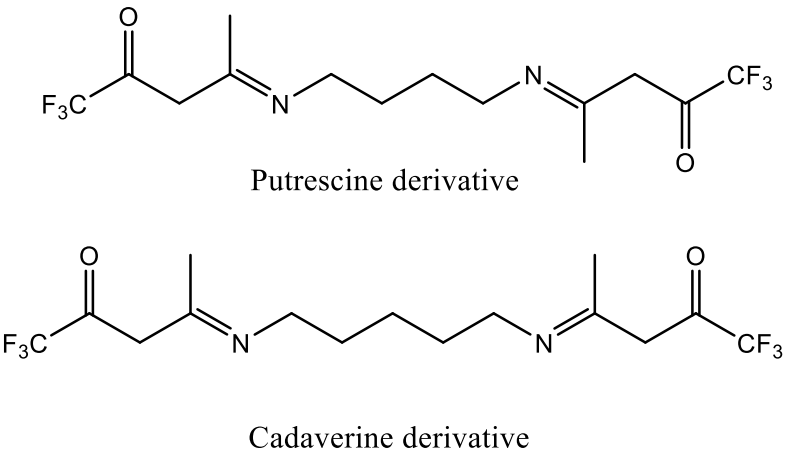


Figure 5.6 Structural diagram of diamine derivatives with TFAA

These finding were previously reported (Awan *et al*., 2008). The 54 amu difference between these observed molecular ions agreed with previous reported values (Khuhawar *et al*., 1999). It has been suggested that a cyclic adduct is formed involving three water molecules (Awan *et al*., 2008). The carbonyl qroups in the diamine derivatives can react with water molecules because of two lone pairs on the oxygen. Hydrogen bonding could be involved in this mechanism.

The derivatization experiment was performed in the organic phase using ethanol. However, as ethanol is a solvent that kills bacteria, it is essential to use another solvent in this experiment to preserve the bacteria. And because NMP is the solvent used to prepare the enzyme substrates it was selected for further investigation. The putrescine and cadaverene derivatives were detected by GC/MS at the same retention times and with the same peak intensity and same mass spectra. Therefore, the results concluded that the derivatization reaction for putrescine and cadaverene with TFAA can be obtained using NMP.

5.2.4.2 Evaluation of headspace (on-fiber) derivatization

This experiment was carried out as described previously by Awan *et al*. (2008) to investigate the presence of putrescine and cadaverine derivatives in the headspace of the samples. The derivatization and extraction occurred at the same time in the headspace (Section 3.9.2.4.2). The sampling method (Section 5.2.4.1) was direct injection of the reaction product into the GC. However, in this experiment the sampling was performed using HS-SPME with an 85 µm polyacrylate (PA) fiber. Although the sampling method was different, the reaction conditions were still the same, and the derivatized compounds were detected at similar retention times (Figure 5.7). This indicated that this method could successfully extract putrescine and cadaverine derivatives from the headspace of the sample using a polar SPME fibre. This method was then ready to adopt to extract putrescine and cadaverine as derivatives, after reacting with TFAA in solution (broth).

To apply this test to the proposed *Salmonella* detection method this derivatization reaction must be performed in an aqueous phase (broth). A 10 mL broth solution was used as a liquid culture media for *Salmonella*. Therefore, an investigation into the derivatization reaction in 10 mL broth was done. However, unfortunately no putrescine and cadaverine derivatives were detected. Therefore, a different type and volume of broth and distilled water was investigated.

Table 5.2 summarizes the results. The putrescine and cadaverine derivatives were detected only when using 1 mL of TSB and when using 1 mL of distilled water. However, this volume was not enough to perform the growth of *Salmonella* samples as it will not contain enough nutrient for *Salmonella* to grow and produce enzymes that hydrolyse the added substrates. In addition, there was no reaction detected in 1 mL RVS broth which is the selective *Salmonella* broth that was planned for use with the food samples.

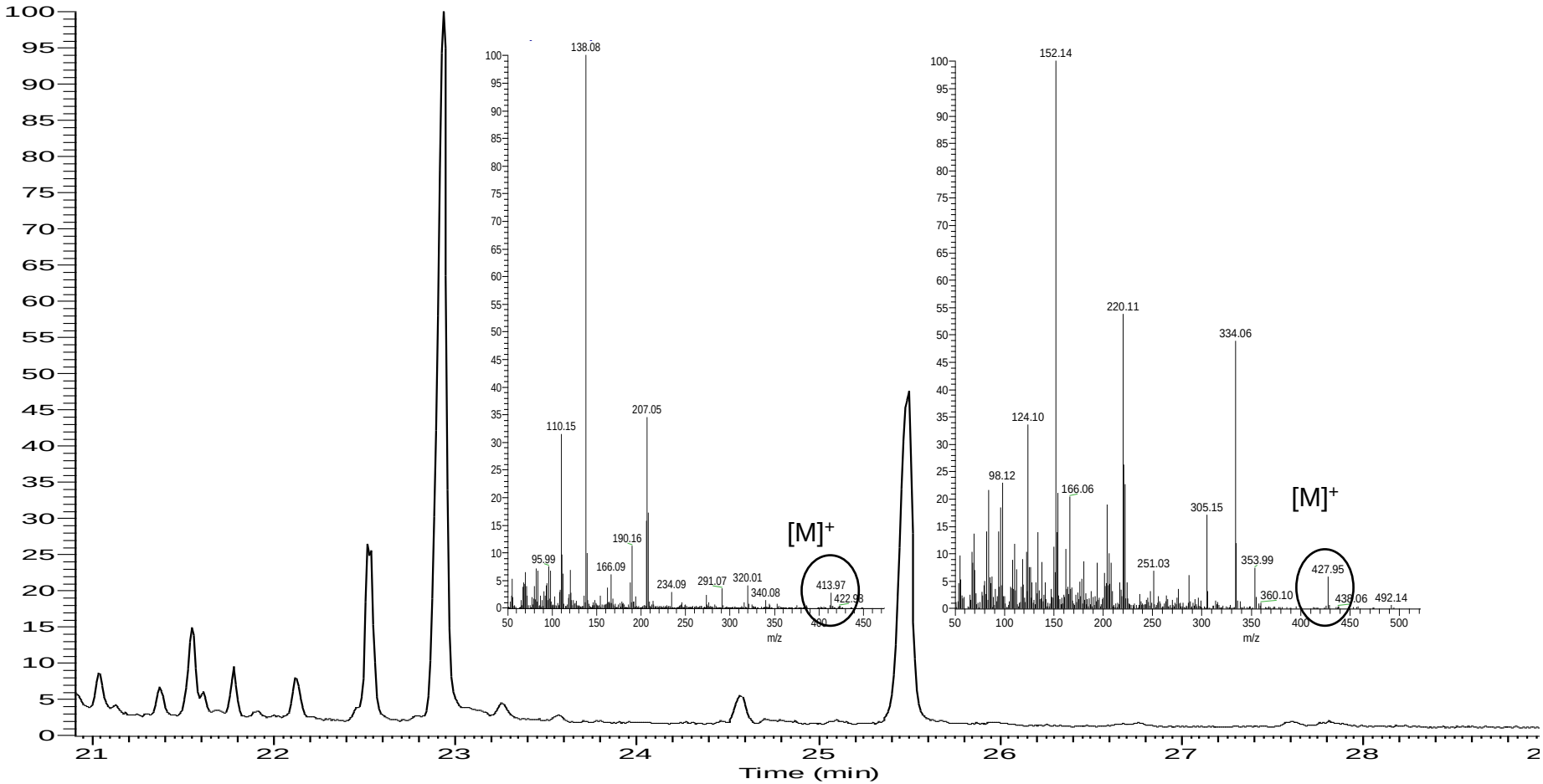


Figure 5.7 The chromatogram of putrescine (t_R 22.9 min) and cadaverine (t_R 25.5 min) derivatives) and the mass spectrum of putrescine derivative and the mass spectrum of cadaverine derivative analysed with nonpolar GC column and polar SPME fibre (PA)

After a review of the information in the literature (Conte and Miller, 1996), a study to investigate the effect of pH on the derivatization reaction to potentially overcome the difficulties of detecting putrescine and cadaverine derivatives in 10 mL broth was done.

Table 5.2 Investigation study of headspace (on-fiber) derivatization

Volume of solution	RVS broth	TSB broth	H_2O
10 mL	ND	ND	ND
5 mL	ND	ND	ND
1 mL	ND	D	D

ND = no putrescine and cadaverine derivatives detected, D = putrescine and cadaverine derivatives detected

5.2.4.3 Effect of the pH on the aqueous phase derivatization and headspace extraction

The derivatization reaction of putrescine and cadaverine have been previously examined (Khuhawar *et al*., 2000) over the pH range 3 to 10 using different buffer solutions. The study observed that a reasonable extraction was detected at pH 6.75. Therefore, phosphate buffer pH 7 and pH 10 were used in this experiment for potential achievement to a successful aqueous phase derivatization. The selective *Salmonella* RVS broth and TSB were used in this experiment to perform the derivatization reaction. After adjusting the pH of 10 mL TSB, 10 mL RVS and 10 mL distilled water to 6.75 the results showed no derivatives being detected. Therefore, an investigation into the reaction in different volume (1, 5, 10 mL) of RVS and TSB was carried out. These broth volumes were tested without adding buffer and were repeated after the pH adjusted to 7 and 10, with phosphate buffer. Table 5.3 summarises the results of these experiments. The cadaverine and putrescine derivatives were detected only in the headspace of the 1 mL samples of TSB at pH 7 and 10. The other

volumes 5 and 10 mL of TSB and all tested volume of RVS showed no cadaverine and putrescine derivatives in the headspace.

Table 5.3 Headspace extraction and investigation study of aqueous phase derivatization for cadaverine and putrescine standards

Phosphate buffer	TSB			RVS		
	1 mL	5 mL	10 mL	1 mL	5 mL	10 mL
pH 7	D	ND	ND	ND	ND	ND
pH 10	D	ND	ND	ND	ND	ND
Without buffer	D	ND	ND	ND	ND	ND

ND = no putrescine and cadaverine derivatives detected, D = putrescine and cadaverine derivatives detected

Other experimental variables such as the order of adding the reagents was investigated and again there was no detectable derivatives. Adjusting the pH of 5 and 10 mL RVS, TSB and distilled water to pH 6.5, 7.3, 8 and 9 using NaOH (1M) and HCl (1M) did not improve the derivatization reaction. Increasing the concentration of cadaverine, putrescine and TFAA by 10 times whilst keeping the mole ratio of the reactants constant showed no difference in the results and no derivatives were detected in 10 mL of broth or distilled water. As the reagents had been prepared in organic solvent and the reaction in 1 mL TSB was successful, the effect of the organic phase / aqueous phase ratio was investigated in 5 mL and 10 mL broth, once again no derivatives were detected.

Questions have been raised about the occurrence of the derivatization reaction in the aqueous phase due to the absence of cadaverine and putrescine derivatives in the headspace when using 10 mL broth or water. For more understanding of the investigation to the presence/absence of putrescine and cadaverine derivatives in solution (broth) a further study is done.

5.2.4.4 Evaluation of aqueous phase derivatization and solvent extraction

The purpose of this experiment was to investigate the presence of putrescine and cadaverine derivatives in the solution (broth) as products of the successful derivatization reaction. The derivatization reaction of putrescine and cadaverine with TFAA was performed in 5 mL and 10 mL TSB and in 1 mL, 5 mL and 10 mL RVS broth. The pH of the broths was adjusted to different pH access range the 7-10 using 10 M NaOH. The products of this reaction were conducted using solvent extraction with dichloromethane (DCM) at room temperature. Experimental details can be found in (Section 3.9.2.4.4). The results obtained are summarised in Table 5.4.

Table 5.4 Solvent extraction and the investigation study of aqueous phase derivatization for cadaverine and putrescine standards

Phosphate buffer	TSB		RVS		
	5 mL	10 mL	1 mL	5 mL	10 mL
pH 7	D	ND	ND	ND	ND
pH 10	D	ND	ND	ND	ND
Without buffer	D	ND	ND	ND	ND

ND = no putrescine and cadaverine derivatives detected, D = putrescine and cadaverine derivatives detected

Cadaverine and putrescine derivatives were extracted from 5 mL TSB, while no putrescine and cadaverine derivatives were detected in any of the volumes of RVS broth. The results of this investigation showed that the derivatization reaction in RVS with these conditions (pH 7 and 10) was not successful neither in the headspace nor in aqueous phase. These results needed more investigation. An implication of this is the possibility that this reaction might need more basic pH medium than pH 10 as the pKa values for the amino groups in putrescine are 9.35 (+2) and 10.92 (+1) and for cadaverine are 10.05 (+2) and 10.92(+1) in aqueous solution (Conte and Miller, 1996). Therefore, in the pH

range 7-10 these compounds exist as protonated diamines and the protonated form of the diamine needed to be converted to the free base form to conduct the derivatization reaction. This can be done by using a higher pH solution than 10, for example pH 11-12, and subsequently the derivatization reaction would be performed between the diamines and the reagent TFAA under these conditions. In respect to this, another investigation of the headspace (on-fiber) derivatization of standard cadaverine and putrescine at a pH higher than 10 was carried out. The derivatization reaction of putrescine and cadaverine standards with the reagent TFAA was performed (Section 3.9.2.4.2), with adjusted the pH of 10 mL TSB and 10 mL RVS to pH 10.5, 11, 12 using 10 M NaOH. The results showed detectable peaks for putrescine and cadaverine derivatives in the headspace of the samples. The pH 12 and 13 showed more intense peaks of both derivatives than pH 11. Figure 5.8 shows the detection of cadaverine derivatives at pH 12.

It has been demonstrated in this experiment that the analysis and the detection of cadaverine and putrescine derivatives can be performed in headspace of 10 mL, pH 12 RVS broth. This successful result and the new experimental conditions (pH 12 of 10 mL broth) could be applied to *Salmonella* samples to investigate the presence of cadaverine and putrescine in the *Salmonella* sample as a result of decarboxylase activities. The next Section will examine the findings of this derivatization method on *Salmonella* samples.

5.2.4.5 Evaluation of cadaverine and putrescine derivatives in *Salmonella* samples

This Section applies the conditions of the successful derivatization reaction, obtained in the previous Section, to detect *Salmonella* decarboxylase activities. *Salmonella* samples were prepared and analysed on GC/MS (Section 3.9.2.4.5). The headspace analysis and the solvent extraction (Section 3.9.2.4.4)

of the *Salmonella* samples showed no detectable peaks for putrescine and cadaverine derivatives. To assess this investigation, it would be reassuming to see some data from controls which may give some ideas about this reaction.

Therefore, *Salmonella* samples spiked with cadaverine and putrescine were prepared and analysed for cadaverine and putrescine derivatives in the same manner. Spiked *Salmonella* samples with 0.5 mL, 1 M cadaverine and putrescine showed no detectable derivatives peaks when using headspace extraction but, detectable derivatives peaks when using solvent extraction. This means cadaverine and putrescine found in solution of spiked *Salmonella* sample react with the reagent TFAA to produce putrescine and cadaverine derivatives. The derivatives product could not be present in the headspace but could be extracted from the solution. The absence of the cadaverine and putrescine derivatives in the headspace of *Salmonella* samples could be due to the level of growth and the amount of the enzyme produced or could be due to the un-favourable reaction conditions.

5.2.4.6 Evaluation of glucose level on *Salmonella* growth and production of lysine and ornithine decarboxylase

Salmonella, as all *Enterobacteriaceae,* cause an initial fermentation of glucose-producing acid that creates an acidic environment. In response to this acidity *Salmonella* produce lysine and ornithine decarboxylase and form cadaverine and putrescine. Therefore, an investigation into *Salmonella* samples that were grown in TSB and not selective RVS, with addition of 1 % - 3 % glucose to increase a source of energy for *Salmonella* growth was carried out. The results of this experiment showed no cadaverine and putrescine derivatives in both headspace and solution.

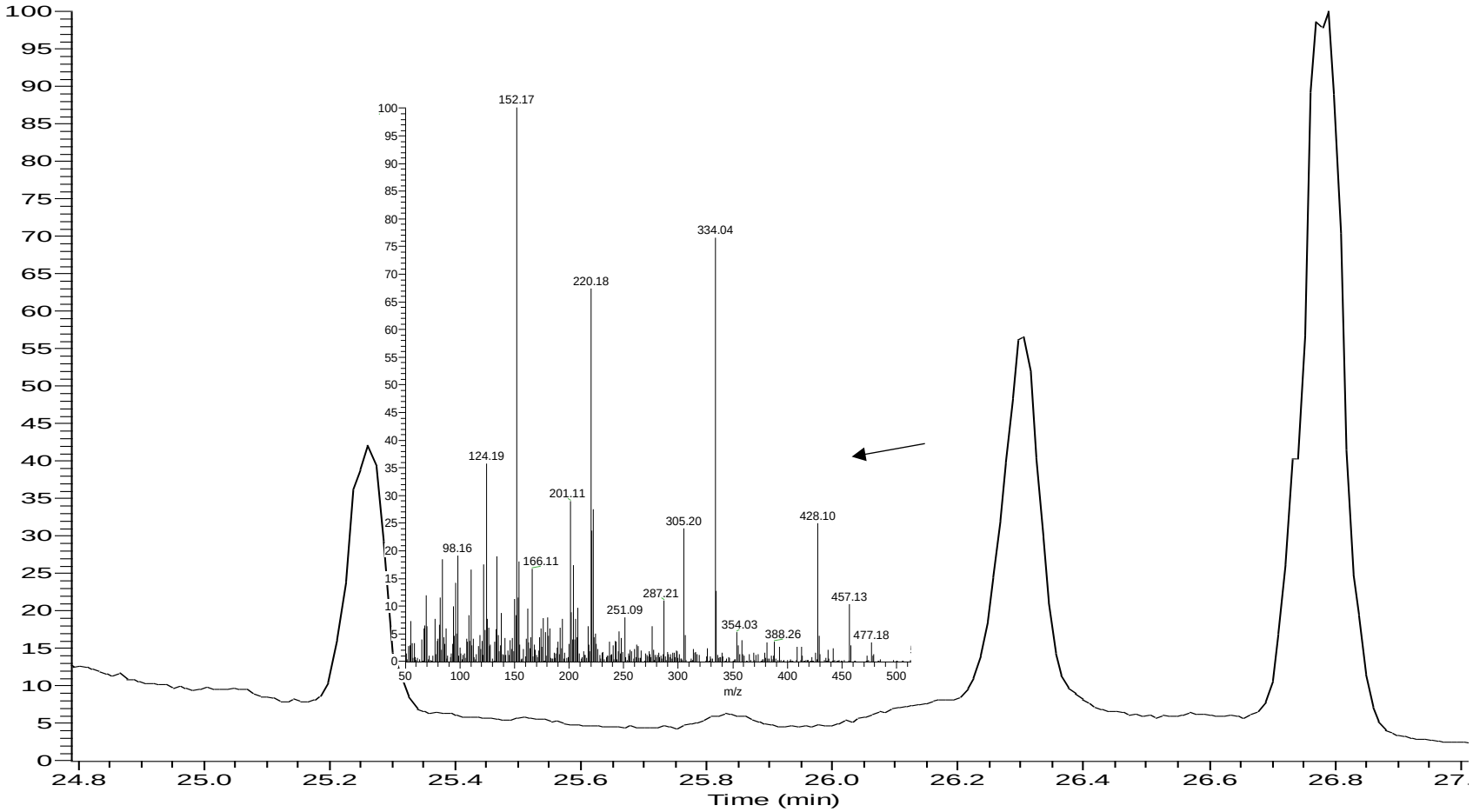


Figure 5.8 Cadaverine derivatives of pH 12 using HS-SPME GC/MS

A specific decarboxylase media was used to ensure the production of cadaverine and putrescine in *Salmonella* samples. Falkow`s lysine decarboxylase modified broth was prepared and used to prepare *Salmonella* samples (Section 3.9.3.4.6). The derivatization results shown no cadaverine and putrescine derivatives in the headspace of *Salmonella* samples. Justification to this investigation could be, the decarboxylation is irreversible and usually requires a coenzyme such as pyridoxal phosphate which further enhances decarboxylase activity (Macfaddin, 1987). In addition, the conventional lysine and ornithine decarboxylase test is performed under oil; this is because the breakdown of the amino acids (the lysine and ornithine substrates) occurs anaerobically (Macfaddin, 1987). Both cadaverine and putrescine are stable when produced under anaerobic conditions. Use of the mineral oil in the conventional carboxylase method allows entrapment of the volatiles cadaverine and putrescine. However, it is not convenient in the case of headspace-analysis to perform this test under anaerobic conditions (overlay with mineral oil). It would be useful to perform the conventional decarboxylase method using a pH indicator with and without mineral oil under identical conditions to investigate the production and presence of cadaverine and putrescine in *Salmonella* samples.

Ultimately, after investigations to the cadaverine and putrescine derivatization reactions and the investigations to the presence of cadaverine and putrescine in *Salmonella* samples, performing the conventional decarboxylase test in *Salmonella* samples parallel with the positive control would be time consuming. As the cadaverine and putrescine derivatives were not detected neither in the headspace nor in the extracted solution of *Salmonella* samples the use of decarboxylases as markers for *Salmonella* is impossible. Therefore, the detection of *Salmonella* decarboxylases activity was removed from the targeted

enzymes activity proposed as a detection/identification tool for *Salmonella* in food samples.

Preliminary investigations and application to the proposed detection approach is carried out by incorporation of the successfully tested enzyme substrates into growth media and detection of *Salmonella* enzymatic activities in the headspace by detecting the liberated VOCs using GC/MS.

5.3 Application of optimised method (Intra and Inter study)

Phenyl α-D-galactopyranoside, 2-nitrophenyl octanoate and L-pyrrollidonyl fluoroanilide were the selected successful substrates used to optimise the detection method. The selective *Salmonella* broth RVS was used in the optimisation study. *S. london* and *S. typhimrum* were used as representative of *Salmonella* species. The preparation of *Salmonella* samples was as described in Section 3.6. and the analysis of the VOCs as described in Section 3.9. The VOCs detected in *Salmonella* samples were phenol and 2-nitrophenol. The substrate L-pyrrollidonyl fluoroanilide did not hydrolyse in the samples; *Salmonella* is known to be negative for PYRase. So, an investigation into the amount of phenol and 2-nitrophenol liberated from the same *Salmonella* sample was done (intra effect study). Secondly, the VOCs liberated from 3 different *Salmonella* samples was done (inter effect study). The VOCs liberated were quantified and the amount of the VOCs are presented in Table 5.5.

In the intra effect study *S. london* and *S. typhimrum* were analysed 3 times (n = 3) in an hour and a half each, as each run need half an hour. The amounts of 2-nitrophenol and phenol released by *Salmonella* enzymatic activities after overnight incubation increased gradually with time. This could be due to the nature of the biological reactions. For example, production of the enzymes and

the growth level of the *Salmonella* strains. In addition, in the inter effect study the concentration of both VOCs detected in both samples (n = 3) varied in quantity. Therefore, one can conclude from these results, that the amount of VOCs detected in *Salmonella* samples due to enzymatic activities is not important as it is the absence or presence of the VOC signal that explains the presence (or not) of enzymatic activity in the sample.

Table 5.5 The intra and inter effect of detected VOCs during *Salmonella* enzymatic activities

Sample	Intra effect study		Sample	Inter effect study	
	2-Nitrophenol (µg/mL)	Phenol (µg/mL)		2-Nitrophenol (µg/mL)	Phenol (µg/mL)
S. london-1	1.66	7.44	*S. london*-1	1.66	7.44
S. london-1	2.79	9.73	*S. london*-2	1.79	8.32
S .london-1	3.49	9.70	*S. london*-3	2.60	5.46
S. typhimrum-1	0.89	13.6	*S. typhimrum*-1	1.03	13.63
S. typhimrum-1	1.03	15.2	*S. typhimrum*-2	0.73	25.67
S. typhimrum-1	1.22	16.1	*S. typhimrum*-3	0.33	18.71

As this study was targeted to a food application, testing the developed approach against other relevant bacteria is carried out and the results discussed in the following Section.

5.4 Application of approach against other pathogenic

This Section has investigated the *Salmonella* enzymatic assay developed in this chapter against some Gram-positive and Gram-negative bacteria commonly isolated from food. As the enzymes selected for the *Salmonella* detection approach are found in other bacteria, detection of the enzymes activities in some relevant species help achieves high specificity to the method

by adding appropriate selective agents to the growth media and inhibit the growth of such undesirable bacteria. In addition, the selective enrichment broth RVS used in this study was expected to inhibit the growth of Gram-positive bacteria. Knowing what other organisms might be expected to be recovered from this selective medium also increases the specificity in the same manner. The bacteria encountered in the study including some food related pathogenic species of *Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa* and *Campylobacter Jejuni.* These bacteria were grown in both the *Salmonella* selective RVS broth and TSB, for a comparison study to the growth level and the enzymatic production in both culture media. The bacterial samples were prepared, as in Section 3.6.2, with addition of the enzyme substrate, as in Section 3.9.2. The positive enzyme activities of these species were measured by detection of the VOCs phenol, 2-nitrophenol and 3-fluoroaniline. The bacterial samples were analysed after overnight incubation at 37 ºC and the amount of the detected VOCs and the growth level of each organism are summarised in Table 5.6.

All the strains of the bacteria tested showed good growth in TSB while, in the *Salmonella* selective RVS broth, the Gram-positive *Listeria monocytogenes* showed no growth and this is an expected result as the selective enrichment broth RVS is designed to inhibit many Gram-positive bacteria by containing malachite green (Rappaport *et al*., 1956; Schothorst and Renaud, 1985). Both strains of *Listeria monocytogenes* were grown in TSB but no VOC was detected; it was anticipated that no enzyme substrates were hydrolysed. However, Barclay *et al*. (1989) reported esterase activity associated with some *Listeria monocytogenes* strains. Not detecting the 2-nitrophenol in *Listeria monocytogenes* samples could be due to the absence of the C-8 esterase in these strains.

Gram-negative *Campylobacter Jejuni* is a microaerophile that requires oxygen to survive, but requires environments containing lower levels of oxygen than are present in the atmosphere and also it is a capnophile, requiring an elevated concentration of carbon dioxide (Dias-Wanigasekera, 2011). These bacteria showed no growth in RVS; this was investigated using the plate culture method and no colonies were observed after overnight incubation of this sample on tryptone soya agar plates. *Campylobacter Jejuni NCTC 11322* hydrolysed the L-pyrrollidonyl fluoroanilide when cultured in TSB and liberated 0.25 µg/mL 3-fluoroanaline indicating the presence of PYRase activity. Colomina *et al*. (1996) reported the presence of C-8 esterase and the absence of α-galactosidase in some *Campylobacter jejuni*. However, both these activities were not detected in this study for this strain. This could be due to the variable reactivity of the species of this strain.

The 2 *Pseudomonas aeruginosa* strains tested were grown normally in both RVS and TSB. Both strains were able to hydrolyse the substrate L-pyrrollidonyl fluoroanilide in TSB liberating 3-fluoroanaline. Whereas in RVS only *Pseudomonas aeruginosa* NCTC 10662 was able to hydrolyse L-pyrrollidonyl fluoroanilide and liberate 0.16 µg/mL 3-fluoroanaline. In the Gram-positive *Pseudomonas aeruginosa* NCTC DSMZ *19980* no PYRase activity was detected and that could be due to the insufficient growth in the selective RVS broth. No C-8 esterase and no α-galactosidase activities were detected in both *Pseudomonas aeruginosa* strains in both media. Freydiere and Gille (1991) reported positive C-8 esterase activity to one of three *Pseudomonas* isolates. Therefore, the tested *Pseudomonas aeruginosa* strains could have a negative C-8 esterase as some of these bacteria are negative.

In addition, *Pseudomonas aeruginosa* was reported to be negative for α-galactosidase (Kämpfer *et al*., 1991). *Escherichia coli* strains show good growth in both TSB and RVS broth, except *E. coli* NCTC 10218 which showed poor growth in RVS; this was proven by the plate count method, as no colonies observed after overnight incubation. In addition, indole production is a common diagnostic marker for the growth, presence and for identification of *Escherichia coli* in a sample (Wang *et al*., 2001). The indole peak was detected at 19.9 minutes (Figure 5.9) in all *E. coli* samples except *E. coli* NCTC 10218. The phenol peak was detected in all *E. coli* strains samples, generated by cleavage of phenyl α-D-galactopyranoside; except in *E. coli* 10418 samples. These results indicated the positive α-galactosidase activity *of E. coli* strains, this was as expected and previously reported (Kämpfer *et al*., 1991). No C-8 esterase activity was detected in all *E. coli* strains tested, and the negative C-8 esterase has been reported (Dealler *et al*.1992). Similarly, no PYRase activity was observed in *E. coli* samples and this negative activity have been reported previously (Freydiere and Gille, 1991; Chagla *et al*., 1993).

The present experiment was designed to determine the specificity of the developed detection method and the selectivity of the growth media RVS. The selective enrichment broth RVS found to inhibit the growth of most Gram-positive bacteria. However, RVS broth didn't suppress the growth of *Pseudomonas aeruginosa*. In addition, some strains of *E. coli* were recovered from this broth. It may be that because the initial level of these bacteria (10^6 CFU/mL) are present in higher than what can be inhibited by RVS broth. The results, as shown in Table 5.6, indicate that presence of one strain of *E. coli*, *Pseudomonas aeruginosa* or *Listeria monocytogenes*, or *Campylobacter Jejuni* show no interfere with the identification of *Salmonella* as none of these bacteria produce 2-nitrophenol and

phenol. However, the presence of two different species that produce phenol and 2- nitrophenol could lead to detection of false positive results. As food samples are one of the heavily contaminated materials and the presence of different bacteria in food sample is expected inclusion of a suitable inhibitor agent could be needed to accomplish this task. For example, addition of suitable antibiotics to increase the selectivity and the specificity of the detection method.

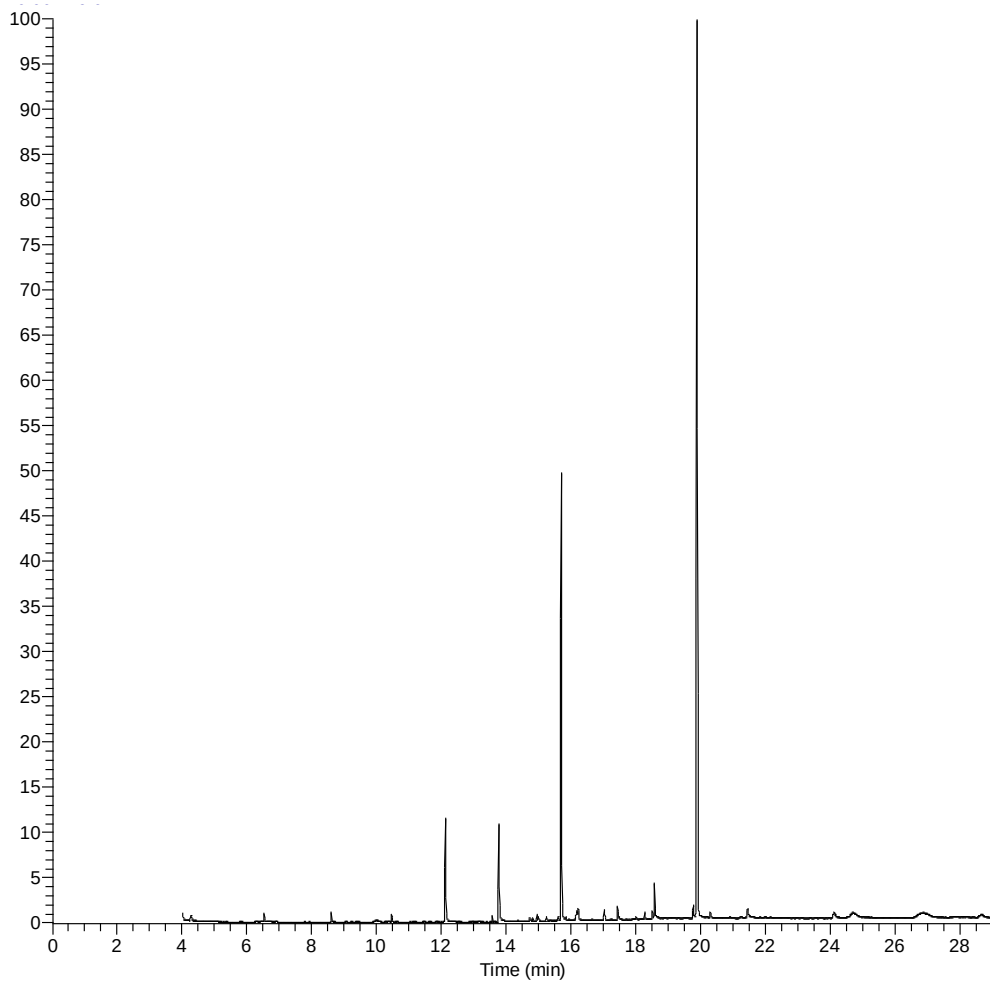


Figure 5.9 VOCs detected on *E. coli* 10213 using HS-SPME GC/MS. (t_R = 12.12 min is NMP, t_R = 13.8 min is 2-nitrophenol (blank), t_R = 15.7 is phenol, t_R = 18.6 min is 2-nitrophenyl octanoate, t_R = 19.9 is indole, other peaks are back ground noise from the SPME fiber and the broth)

Another parameter that is useful to study when studying bacterial enzyme substrate activity is the effect of the amount of organic solvent (NMP) used to prepare the stock solution of the substrates.

Table 5.6 Enzyme substrates activities and VOC profiles of common food related pathogens

Bacteria	Media	Growth level	2-Nitrophenol µg/mL	Phenol µg/mL	3-Fluoroaniline µg/mL
Listeria monocytogenes NCTC 11994	RVS	-	ND	ND	ND
	TSB	+	ND	ND	ND
Listeria monocytogenes NCTC 105376	RVS	-	ND	ND	ND
	TSB	+	ND	ND	ND
E coli NCTC 10418	RVS	-	ND	ND	ND
	TSB	+	ND	48.93	ND
E coli NCTC K12	RVS	+	ND	1.66	ND
	TSB	+	ND	2.57	ND
E coli NCTC 18039	RVS	+	ND	7.4	ND
	TSB	+	ND	4.16	ND
E coli NCTC 10213	RVS	+	ND	19.03	ND
	TSB	+	ND	3.35	ND
E coli O157: H	RVS	+	ND	2.46	ND
	TSB	+	ND	1.85	ND
Pseudomonas aeruginosa NCTC DSMZ 19980	RVS	+	ND	ND	ND
	TSB	+	ND	ND	0.53
Pseudomonas aeruginosa NCTC 10662	RVS	+	ND	ND	0.16
	TSB	+	ND	ND	0.16
Campylobacter Jejuni NCTC 11322	RVS	-	ND	ND	ND
	TSB	+	ND	ND	0.25

- = no growth; + = normal growth; ND = not detected.

5.5 *Salmonella* susceptibility in N-Methyl-2-pyrrolidone (NMP)

Microorganisms live, grow, divide, and function in aqueous media however, in some cases it has been observed that some bacteria are able to survive in the presence of small amounts of organic solvent (Rajagopal, 1996).

It is clear that there are substantial differences among bacterial cells in their susceptibility and reaction to organic solvents. Clearly the effect of the amount of solvent such as N-methyl-2-pyrrolidone (NMP) on the growth of bacteria (Gram-negative and Gram-positive) is different for each different species and each organism may exert varying susceptibility (Křížek *et al*., 2015). The solvent NMP is a very strong solubilizing agent that has been used to prepare enzyme substrates used in this study. Thus it becomes essential to ensure that the final concentration of the organic solvent NMP used is not likely to interfere with the growth of *Salmonella* strains in the samples.

The effect of NMP concentrations on the growth of *Salmonella* was investigated. The experimental result (Figure 5.10) showed that NMP can be used to solubilize the substrate at low concentration and can be used up to a maximum of 0.5 % without any issue reported. As can be seen (Figure 5.10) an increase in the amount of the solvent NMP in the sample decreased the maximal growth of *Salmonella* strains. Therefore, the recommended amount of NMP that can be used for *Salmonella* growth is between 0.1 and 0.5 % (v/v).

Identification of *Salmonella* contamination sources are vital for immediate action of emergency responders. Therefore, the developed approach and using enzyme substrate and VOCs analysis for *Salmonella* detection has been made

to shorten the overall analysis time while increasing specificity of *Salmonella* detection.

5.6 Time study

2-Nitrophenol (0.724 ± 0.06 µg/mL) and 2-chlorophenol (0.15 ± 0.02 µg/mL) as indicative of C-8 esterase activity were liberated by *S. stanley* samples after 4 hours incubation at 37 ºC.

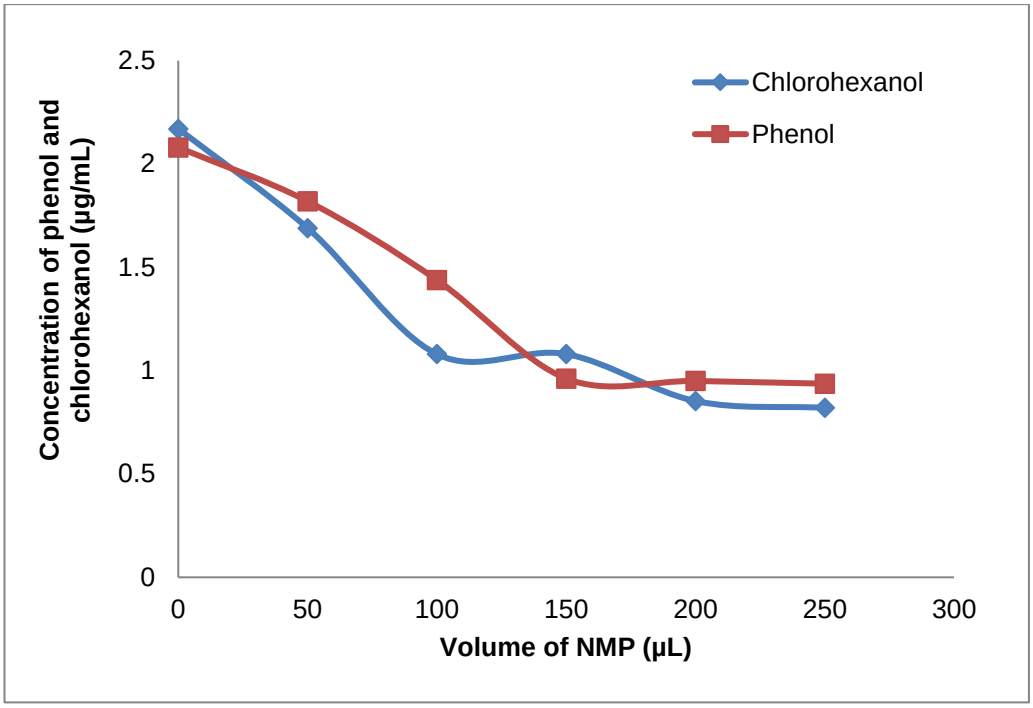


Figure 5.10 Effect of NMP on *Salmonella* growth

However, liberated phenol (0.10 ± 0.14 µg/mL) was detected after 5 hours incubation at 37 ºC. Figure 5.11 shows the liberated VOCs over a 24-hour time period. The enzymatic activities of *S. stanley* together were detected after 5 hour (at an incubation at 37 ºC). Therefore, the results of the study indicated that the VOCs liberated during enzymatic activities of *Salmonella* could be detected after the fifth hour of incubation. The signal and the amount of the VOCs detected increased gradually due to the increasing number of cells of *Salmonella,* with time,

until the level of *Salmonella* reach 10^8 CFU/mL. At that point no increase was observed in the amount of VOCs (Figure 5.11).

5.7 The sensitivity of the VOC method

The sensitivity of the method was assessed in terms of initial inoculum size, using phenyl α-D-galactopyranoside and 2-nitrophenyl octanoate. Secondly, using phenyl α-D-galactopyranoside and 2-chlorophenyl octanoate. The initial inocula were prepared (Section 3.6.2) and the VOCs detected via HS-SPME GC/MS after overnight incubation at 37 ºC. The samples were tested in triplicate. It can be seen from the data in Table 5.7 that an initial inoculum of 1–1.5 x 10^0 CFU /mL and 1–1.5 x 10^1 CFU /mL was required for the generation of detectable amount of 2-nitrophenol and phenol, respectively, after overnight incubation at

Table 5.7 Sensitivity of the VOC *Salmonella* detection method

Initial inoculum (CFU / mL	2-Nitrophenol (µg/mL)	Phenol (µg/mL)
1–1.5 x10^0	0.50 ± 0.02	15.5 ± 4.10
1–1.5 x10^1	1.50 ± 0.50	21.5 ± 2.30
1–1.5 x10^2	2.70 ± 0.30	22.4 ±2.60
1–1.5 x10^3	2.90 ± 0.50	24.4 ± 2.70
1–1.5 x10^4	3.01 ± 1.20	25.3 ± 4.70
1–1.5 x10^5	3.40 ± 0.50	29.7 ± 5.40

37 º C the VOCs liberated by *S. stanley* demonstrated that contaminated food samples with at least 1–1.5 x10^0 CFU /mL of *Salmonella* prior to overnight incubation could be detected via detection of the VOCs liberated following enzyme substrate hydrolysis. *Salmonella* are not detectable in certain samples that contain small numbers of organisms (Fricker, 1987) using the standard laboratory procedure, however, the developed method shown to be more rapid and sensitive for detection of *Salmonella* in the samples. The sensitivity of the detection method using phenyl α-D-galactopyranoside and 2-chlorophenyl

octanoate measured in terms of initial inoculum size. An initial inoculum of 1–1.5 x 10^0 CFU /mL and 1–1.5 x 10^1 CFU /mL was required for the generation of

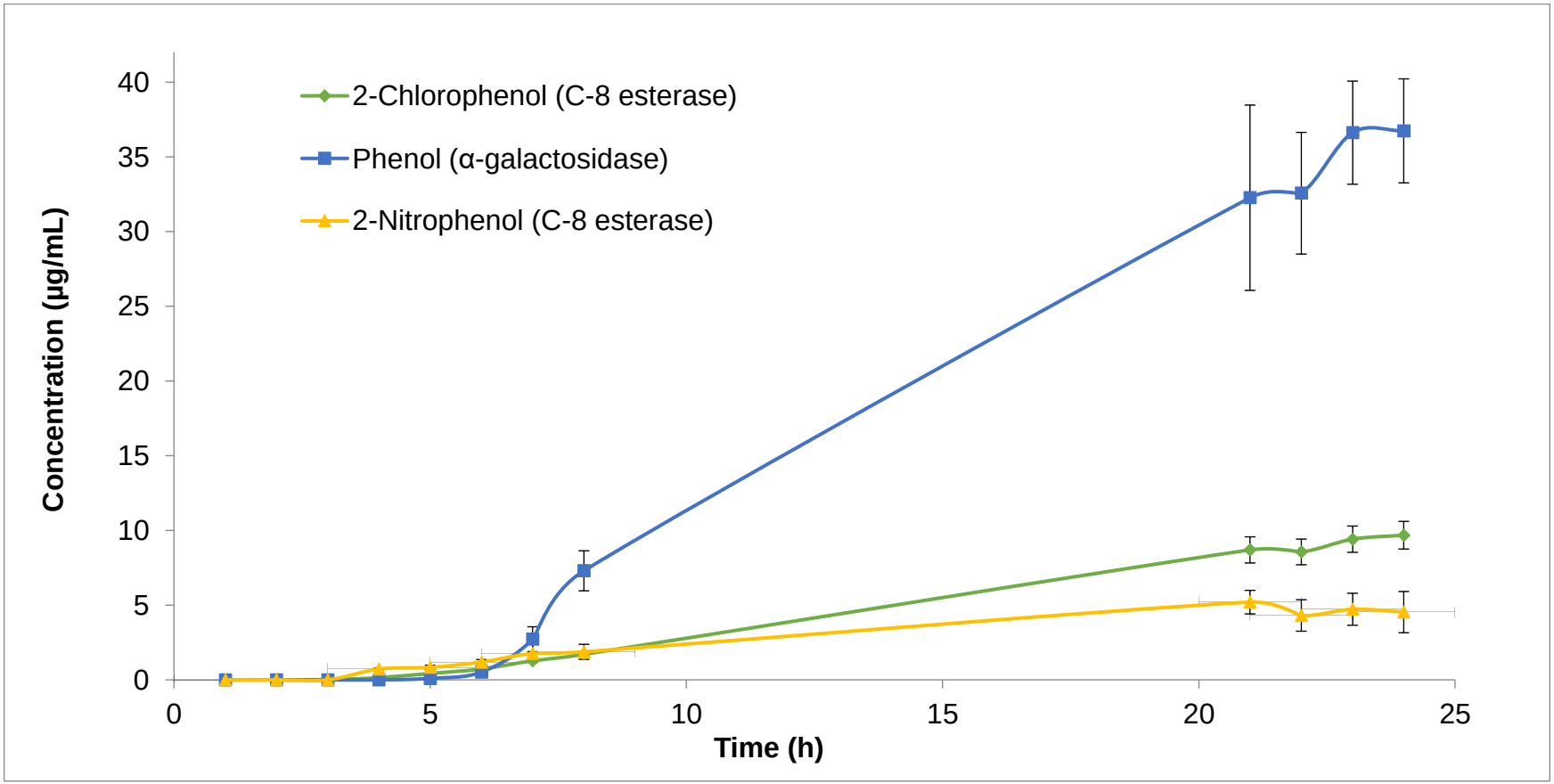


Figure 5.11 Time study scale for enzymatic activity of *S. stanley* in RVS via detection of VOCs

Table 5.8 Sensitivity of *Salmonella* detection method in terms of initial inoculum size

Initial inoculum (CFU / mL)	2-Chlorophenol (µg/mL)	Phenol (µg/mL)
1–1.5 x10^0	0.82 ± 0.10	14.7 ± 1.40
1–1.5 x10^1	1.20 ± 0.02	15.6 ± 1.64
1–1.5 x10^2	1.60 ±0 .17	16.5 ± 0.96
1–1.5 x10^3	1.66 ± 0.06	25.2 ± 0.96
1–1.5 x10^4	1.70 ± 0.17	26.5 ± 2.20
1–1.5 x10^5	1.90 ± 0.16	26.2 ± 1.90

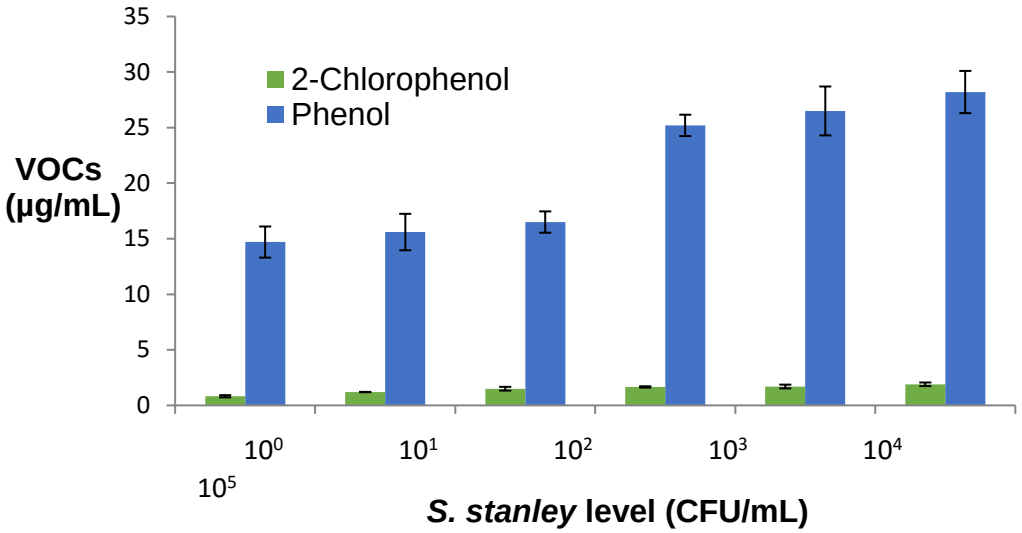


Figure 5.12 Sensitivity of *Salmonella* detection method in terms of initial inoculum size

2-chlorophenol and phenol, respectively, after overnight incubation Table 5.8 and Figure 5.12. The sensitivity of the developed *Salmonella* detection method using the analysis technique HS-SPME GC-MS was compared with the Standard Plate Count (SPC) method. The SPC (the total viable count), is one of the tests applied to indicate the microbiological quality of food (Anderson *et al*., 2011). This test is done by plating the *Salmonella* samples on a solid agar and inoculating 10 mL RVS broth with *S. stanley*. The culture plates were prepared using peptone soya (Section 3.6.4). Incubation followed by counting bacteria that grow on plates and headspace detection to the VOCs in the samples. The results are summarised in Table 5.9. After incubation for 5 h and 10 h no colonies were formed on the

plates for sample 10^0 CFU / mL. The conclusion of the VOCs 2-chlorophenol and phenol were 0.82 ± 0.10 and 14.7 ± 1.40 µg/mL, respectively. The same plates showed 40 colonies / mL after overnight incubation and the VOCs phenol and 2-chlorophenol were detected in these samples. Plates inoculated with 10^1 CFU /mL showed growth of *Salmonella* colonies after 10 h incubation. The average number of *Salmonella* colonies observed in these plates is 120 colonies /mL and the VOCs phenol and 2-chlorophenol were detected at the same time in these samples. These results show that the HS-SPME GC/MS is rapid and sensitive to detect *Salmonella* in sample incubated in selective broth than culture method using non selective agar media. Comparing this result with the sensitivity of some other techniques such as culture method and combined immunomagnetic separation-polymerase chain reaction that need 12-h nonselective pre-enrichment to detect *Salmonella* in milk samples (1–10 CFU/mL) (Mercanoglu Taban *et al*., 2009) it raise up an implication of the possible detection of *Salmonella* with contamination level as low as 10^0 CFU/mL in a sample after 10 h incubation in selective broth via the detection of exogenous volatile organic compound metabolites released by enzymatic hydrolysis using HS-SPME GC/MS.

Table 5.9 Comparison of sensitivity of HS-SPME GC/MS *Salmonella* detection method with Standard Plate Count method

Dilution CFU/mL	**5 h incubation**		
	Colony/mL	**2-Chlrorophenol**	**Phenol**
10^0	0	ND	ND
10^1	0	ND	ND
Dilution CFU/mL	**10 h incubation**		
	Colony/mL	**2-Chlrorophenol**	**Phenol**
10^0	0	0.80 ± 0.10	14.7 ± 1.4
10^1	120	1.03 ± 0.40	15.6 ± 1.64
Dilution CFU/mL	**18-24 h incubation**		
	Colony/mL	**2-Chlrorophenol**	**Phenol**
10^0	40	1.5 ± 0.05	21.5 ± 2.6
10^1	120	3.2 ± 0.03	31.6 ± 2.4

ND= not detected

5.8 Summary

The study described in this chapter is an evaluation and investigation into the development of a selective detection method for *Salmonella* in food samples. The selectivity of the detection method was designed by using *Salmonella* selective broth (RVS) as the growth media and use of enzyme substrates that liberate exogenous VOCs. All *Salmonella* strains tested in this study hydrolysed the substrate phenyl α-D-galactopyranoside and generated phenol as a marker of α-galactosidase activity. The investigation of commercial and synthesised C-8 esterase substrates ends up with an assay working for *Salmonella* C-8 esterase test as a marker for *Salmonella* identification and detection in food samples. The absence of liberated 3-fluoroanaline with the synthesised enzyme substrate L-pyrrollidonyl fluoroanilide was a useful indicator for *Salmonella*. In addition, the presence of the liberated VOC with this substrate could be a potential marker for the presence of other pathogenic bacteria.

The difficulties in detecting cadaverine and putrescine derivatives cause withdrawal of the decarboxylase test from the *Salmonella* targeted enzymes. Testing other food related species provided knowledge about some interfering organisms that might be recovered from RVS broth during food analysis. The approach described in this chapter shows potential for future application in food samples to detect and identify *Salmonella* species in food samples of a level as low as 10^0 CFU /mL within a 5 h incubation at 37 ºC by the detection of the liberated VOCs using HS-SPME GC/MS.

Chapter 6: Detection of *Salmonella* in food samples via the detection of exogenous volatile organic compound metabolites released by enzymatic hydrolysis

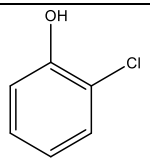
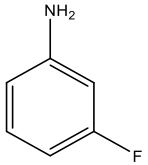
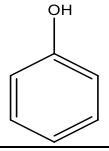
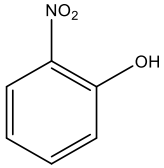
6.1 Introduction

In the last chapter (chapter 5) an approach for detecting *Salmonella* in food samples using enzyme substrates inoculated in culture media was developed. In this chapter the developed *Salmonella* detection method has been applied to a variety of food types considered as the most common sources of *Salmonella.* The *Salmonella* VOCs analysis via hydrolysis of enzyme substrates using HS-SPME GC/MS carried out. Results of these experiments are described and discussed here. Further optimisation as a *Salmonella* detection method have been done and are discussed in detail in this chapter. Identification of bacteria isolated from food samples using MALDI-TOF is presented in this chapter.

6.2 Quantification data of liberated VOCs

The amount of liberated VOCs by *Salmonella* strains were quantified using external calibration. Calibration graphs of all VOCs generated by hydrolysis of 100 µg/mL enzyme substrates: phenyl α-D-galactopyranoside, L-pyrrollidonyl fluoroanilide, 2-nitrophenyl octanoate respectively and 2-chlorophenyl octanoate were prepared as described in Section 3.9.1. The calibration graphs of 2-chlorophenol, 2-nitrophenol, phenol and 3-fluoroaniline were prepared by spiking the standard solutions into 10 mL of blank RVS broth. All the VOCs displayed linearity over a five-point concentration with a correlation coefficients exceeding 0.99. The results are shown in Table 6.1.

Table 6.1 Quantitative data for bacterial VOCs

VOC	Structure	Retention time (t_R;min)	Y = mx + c	Correlation coefficient R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
2-Chlorophenol		14.1	4 x10^{06} x + 74460	0.9989	1-50	0.0140	0.0467
3-Fluoroaniline		14.5	1 x10^{06} x - 55299	0.9994	1-50	0.0049	0.0163
Phenol		16.0	52153x + 46858	0.9977	10-100	0.0451	0.1503
2-Nitrophenol		13.8	74909x - 20641	0.9996	1-50	0.0579	0.1929

LOD = limit of detection, LOQ = limit of quantification

6.3 *Salmonella* detection method using 2-nitrophenyl octanoate

Detection of *Salmonella* in a sample relies on the detection of the α-galactosidase activity (+), C-8 esterase activity (+) and pyrrolidonyl peptidase activity (-). The enzyme substrates used are phenyl α-D-galactopyranoside, 2-nitrophenyl octanoate and L-pyrrollidonyl fluoroanilide. These enzyme substrates react with *Salmonella* enzymes to produce the VOCs, phenol and 2-nitrophenol. Detection of these two VOCs and the absence of 3-fluoroanaline is indicative of *Salmonella* contamination.

S. stanley was chosen to be used as a control in the food experiments because it produced a quantification signals and the highest amount of 2-nitrophenol and second highest amount of phenol among the 6 strains tested as detailed in Section 5.2.1 and Section 5.2.3. Moreover, *S. stanley* have been reported as the most common serovar associated with human infections in EU (European Centre for Disease Prevention and Control, 2014) and was among the 20 most frequently reported serovars in other countries (Hendriksen *et al*., 2011). *Salmonella* should not be present in any ready-to-eat product (Regulation, 2007); however raw products like poultry and meat are remarked to have *Salmonella* with an excepted level as low as possible (McEntire *et al*., 2014). The actual number of *Salmonella* in specific food items linked to illness was investigated and estimated to be ranged from tens of organisms to millions (Blaser and Newman, 1982; Roberts *et al*., 1996; Teunis *et al*., 2010). On that basis level of 10^4 CFUs/mL was the chosen level of *S. stanley* to be used as control blank with tested food samples. The three substrates (100 µg/mL) (mentioned above) were tested with *S. stanley* (1 x 10^4 CFU / mL) inoculated in 10 ml RVS broth. The 2-nitrophenol peak was identifiable in the chromatogram at a retention time of 13.8 minutes and it is well separated from the phenol peak (t_R 16 min). The VOC

profile of *S. stanley* is shown in Figure 6.1. The amount of liberated VOCs 2-nitrophenol and phenol were 4.8 ± 1.2 µg/mL and 42.3 ± 4.2 µg/mL, respectively.

This approach shows potential for future use for the detection of *Salmonella* contaminated food. Application of the developed approach have been done and a discussion to of the results given below.

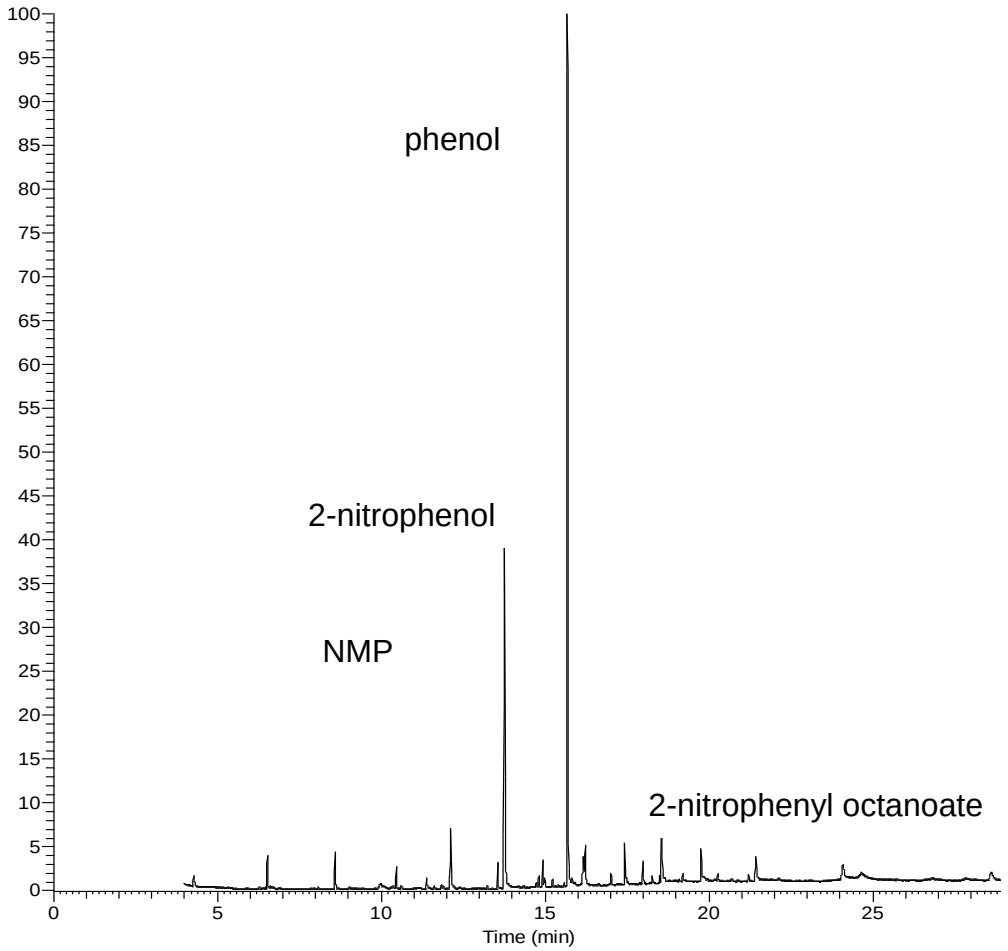


Figure 6.1 VOC profile liberated by *S. stanley* with phenyl α-D-galactopyranoside, L-pyrrollidonyl fluoroanilide and 2-nitrophenyl octanoate

6.3.1 Food applications

The experimental details of food analysis can be found in Section 3.11. In brief, detection of *Salmonella* in foods involve pre-enrichment of the food samples in a non-selective broth buffered peptone water (BPW) for 16-20 h at 37 ºC, this

is to enrich *Salmonella* in the food samples and enable *Salmonella* (if present) to grow to a detectable level. This method is based on ISO 6579:2002. Following the enrichment step, the food samples are then inoculated with the enzyme substrates in *Salmonella* selective broth RVS and incubated overnight at 37 ºC. The liberated VOCs in respect to enzyme substrates hydrolysis were extracted by HS-SPME followed by separation and identification by GC/MS. Spiked RVS samples contain enzyme substrates with *S. stanley* were used as a control blank and un-spiked RVS samples served as a negative control.

All food samples were tested in triplicate and the generated VOCs by un-spiked and spiked samples are shown in Tables 6.1- 6.4. Detection of 2-nitrophenol and phenol in the tested food samples indicates the presence of *Salmonella* while absence of the two VOCs indicates the absence of *Salmonella*. Detection of 3-fluoroanaline points to the presence of other PYRase positive pathogens, that hydrolyse the substrate L-pyrrollidonyl fluoroanilide.

No VOCs were detected in un-spiked semi-skimmed milk, goat milk, full cream milk and cheddar cheese samples. In the artificially contaminated samples (control blank), 2-nitrophenol and phenol were detected as expected. These results indicate that these samples are *Salmonella* free and the proposed *Salmonella* detection method could potentially be used to detect *Salmonella* in selected food samples.

Results presented in Table 6.4 show that chicken samples of skin-on breast fillets and skinless breast fillets produced 2-nitrophenol and phenol, which indirectly signals the presence of *Salmonella* in these chicken samples. However, in two samples 3-fluoroaniline was detected as indicative of PYRase activity. *Salmonella* known to be PYRase negative and this means presence of other

bacteria that are PYRase positive in the samples. Consequently, other bacteria might play a part in the production of 2-nitrophenol and/or phenol detected in these samples. Similarly, in all tested chicken samples and Roquefort cheese, Brie cheese samples and unpasteurized milk samples 3-fluoroaniline was detected as indicative of PYRase activity. Intense indole signals were detected at retention time of 19.9 minutes (Figure 6.2) in these samples (Tables 6.1, 6.2 and 6.4) demonstrated the presence of indole positive bacteria in the samples. Examples include, *E. coli* (Bos *et al*., 2013) and *Proteus vulgaris (O'hara et al., 2000*) where indole serves as a biological marker and is used to differentiate them from other bacteria. However, no isolation and/or identification to any of these bacteria in these samples was carried out due to no previous identification experiment planned. Such a study (i. e. VOC analysis with identification of the isolated pathogenic bacteria recovered from the food samples can be found in the next Section.

In the control blank samples phenyl α-D galactopyranoside substrate reacted with the α-galactoside enzyme of *S. stanley* to produce the VOC phenol. 2-Nitrophenyl octanoate substrate reacted with C-8 esterase enzyme of *S. stanley* in spiked food samples to liberate 2-nitrophenol. Similarly, all spiked samples liberated phenol. However, 2-nitrophenol was not detected in spiked some milk, cheese, eggs and chicken samples. Those are unpasteurised milk sample, and spiked Roquefort and Brie cheese samples, spiked caged hen eggs, and spiked chicken samples of thigh and drumstick, wings and Halal samples. The absence of 2-nitrophenol in the control blanks of these samples could be due to the use of the bacteria present in the food samples due to the nitrogen in the substrate or the nitrogen in the generated 2-nitrophenol as an essential element for growth and source of energy. However, other researchers (Tait *et al*., 2014b)

used the enzyme substrate 2-nitrophenyl-β-D-glucoside and reported the detection of 2-nitrophenol as a result of activity of β-glucosidase in un-spiked unpasteurised milk samples and spiked samples inoculated with *L. monocytogenes* NCTC 11994, when trying to differentiate contaminated milk from non-contaminated milk.

Some other metabolites VOCs have been detected in the studied food samples. For example, 1- decanol have been produced and detected at t_R of 13 minutes in the un-spiked Roquefort cheese samples (Figure 6.2). A previous investigation has shown that *Escherichia coli* and *Klebsiella pneumonia* produce 1-decanol as a metabolic product (Tait *et al*., 2013). Another possible explanation for the detection of such volatiles is that, alcohol compounds are well known to contribute to cheese flavour as Anderson (1965) and Hassan *et al*. (2013) reported that. The absence of the 2-nitrophenol signal in control blank samples led to a substitute in the screening of C-8 esterase substrate in the detection method with a substrate producing more accurate results (positive with the control blank). In this context, it is worthwhile to substitute 2-nitrophenyl octanoate with 2-chlorophenyl octanoate. The next Section will be a discussion to the results of food analysis using 2-chlorophenyl octanoate as C-8 esterase in the proposed *Salmonella* detection method.

6.4 *Salmonella* detection method using 2-chlorophenyl octanoate

In order to improve the *Salmonella* detection method; food analysis was repeated using 2-chlorohpenyl octanoate. The selection of the synthesised 2-chlorophenyl octanoate as C-8 esterase substrate to substitute 2-nitrophenol in the detection method among other synthesised phenolic substrates tested was because 2-chlorophenyl octanoate liberates the second highest amount of VOCs

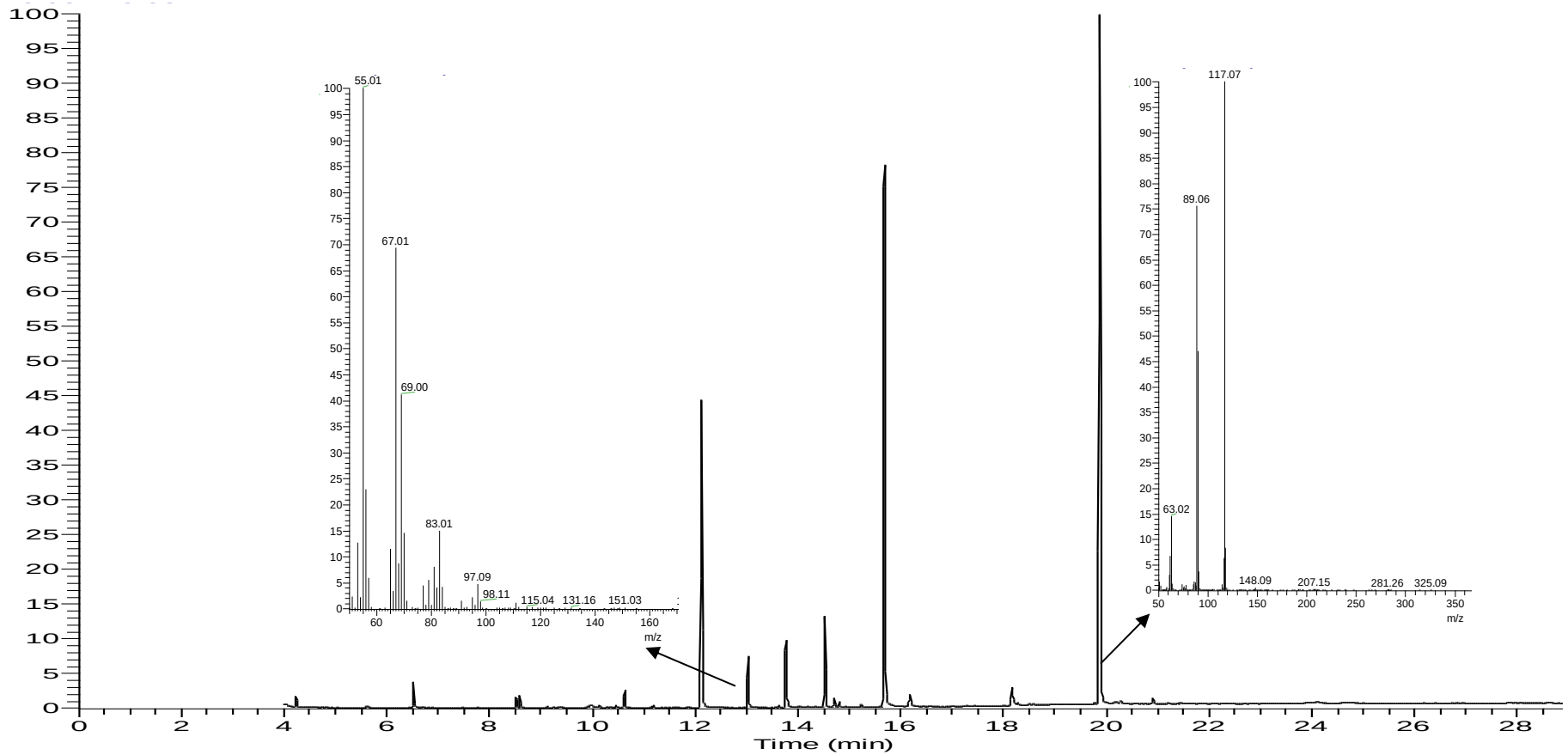


Figure 6.2 VOC profile liberated by Roquefort cheese sample inoculated with phenyl α-D-galactopyranoside, L-pyrrollidonyl fluoroanilide **and 2-nitrophenyl octanoate**

t_R = 12.1 min is NMP, t_R = 13.0 min is 1-decanol, t_R = 13.8 min is 2-nitrophenol, t_R = 14.5 min is 3-fluoroanaline, t_R = 15.7 min is phenol, t_R = 19.9 min is indole

Table 6.2 liberated VOCs by un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU) milk samples detected by HS-SPME GC/MS

VOC (µg/mL)	Whole milk		semi-skimmed milk		Goat milk		full cream milk		unpasteurized milk	
	Un-spiked	Spiked	Un-spiked	Spiked	Un-spiked	Spiked	Un-spiked	Spiked	Un-spiked	Spiked
2-nitrophenol	ND	2.3 ± 0.3	ND	2.3 ± 1.0	ND	7.6 ± 0.9	ND	5.5 ± 1.4	ND	ND*
Phenol	32.5 ± 12	27.3 ± 6.2	ND	3.6 ± 1.0	ND	31 ± 10	ND	1.8 ± 1.5	22.1 ± 3	22.8 ± 3
3-Fluoroaniline	0.84 ± 0.4	0.67 ± 0.4	ND	ND	ND	ND	ND	ND	1.2 ± 0.6	1.2 ± 0.4
Indole	/	/	/	/	/	/	/	/	Yes	Yes

ND = not detected, ND* = positive for *Salmonella* must be detected

Table 6.3 liberated VOCs by un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU) cheese samples detected by HS-SPME GC/MS

VOC (µg/mL)	Roquefort cheese		Brie cheese		Goat milk cheese		Cheddar cheese	
	Un-spiked	Spiked	Un-spiked	Spiked	Un-spiked	Spiked	Un-spiked	Spiked
2-nitrophenol	ND	ND*	ND	ND*	ND	1.1 ± 0.6	ND	0.5 ± 0.5
Phenol	46.3 ± 5.5	41.6 ± 9.8	9.1 ± 0.9	29.1 ± 3.2	2 ± 2.4	4.6 ± 4.2	ND	22.4 ± 4.5
3-Fluoroaniline	0.5 ± 0.8	0.9 ± 0.8	1.8 ± 0.4	2.7 ± 0.3	ND	ND	0.16 ± 0.4	0.14 ± 0.02
Indole	Yes	Yes	Yes	Yes	/	/	/	/

ND = not detected, ND* = positive for *Salmonella* must be detected

Table 6.4 liberated VOCs by un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) eggs samples detected by HS-SPME GC/MS

VOC(µg/mL)	Caged hen eggs		organic eggs		free range eggs	
	Un-spiked	Spiked	Un-spiked	Spiked	Un-spiked	Spiked
2-nitrophenol	ND	ND*	0.63 ± 0.31	1.39 ± 0.73	0.51 ± 0.36	1.89 ± 1.14
Phenol	ND	17.8 ± 3.8	ND	28.8 ± 4.9	ND	36.0 ± 5.05
3-Fluoroaniline	0.19 ± 0.17	2 ± 2.2	0.14 ± 0.0	0.14 ± 0.01	0.19 ± 0.17	0.15 ± 0.006
Indole	/	/	/	/	/	/

ND = not detected, ND* = positive for *Salmonella* must be detected

Table 6.5 liberated VOCs by spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) and un-spiked chicken samples detected by HS-SPME GC/MS

VOC(µg/mL)	skin-on breast fillets		Skinless breast filets		Thigh and drumstick		wings		Halal	
	Un-spiked	Spiked	Un-spiked	Spiked .	Un-spiked	Spiked .	Un-spiked	Spiked	Un-spiked	Spiked
2-Nitrophenol	1.5 ± 0.8	1.3 ± 1.1	1.9 ± 0.4	1.7± 0.5	ND	ND*	ND	ND*	ND	ND*
Phenol	16 ± 0.9	33 ± 12.4	27.3± 1.2	32 ± 2.8	33 ± 8.6	30 ± 11	29 ± 3.2	18 ± 15	24 ± 2.7	21± 1.3
3-Fluoroaniline	2.4 ± 0.9	3 ± 1.7	1.1 ± 0.2	1.2± 0.4	1.3± 0.6	1.4± 0.6	1 ± 0.4	1 ± 0.3	1.5 ± 0.4	2 ± 0.7
Indole	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes

ND = not detected, ND* = positive for *Salmonella* must be detected

after overnight incubation with *S. stanley* at 37 ºC as has been pointed out in Table 6.6. In examining the effectiveness of this substrate, *Salmonella* strains (1-1.5 x 10^6 CFU /mL) were reacted with 100 µg/mL 2-chlorophenyl octanoate in RVS broth. After overnight incubation 2-chlorophenol was liberated and detected in all strains.

Table 6.6 VOCs liberated by *S. stanley* incubated overnight with the substrates

Substrate	VOC	Concentration (µg/mL)
2-Nitrophenyl octanoate	2-nitrophenol	2.01
2-Chlorophenyl octanoate	2-chlorophenol	1.53
2-Methyl phenyl octanoate	2-methylphenol	0.82
2-Chloro-4-methylphenyl octanoate	2-chloro-4-methylphenol	0.30
2,6 Dimethyl phenyl octanoate	2,6 dimethyl phenol	0.15

The 2-chlorophenol peak was easily identifiable on the chromatogram at a retention time of 14.1 minutes (Figure 6.3) and its mass spectrum was shown to be identical to the mass spectrum of standard 2-chlorophenol 10 µg/mL analysed under the same conditions.

The amount of 2-chlorophenol (µg/mL) liberated by *Salmonella* strains was as follows; 0.45 by *S. london*, 1.53 by *S. stanley*, 1.52 by *S. typhimruim*, 1.0 by *S. gallinaruim*, 1.5 by *S. oranienburg*, 0.94 by *S. othmarchen*. The highest quantity of 2-chlorophenol produced by *S. stanley* which made the strain *S. stanley* desirable for further analysis with food samples. The *S. stanley* (1-1.5 x 10^4 CFU /mL) was inoculated in 10 ml RVS broth with 2-chlorophenyl octanoate and phenyl α-D-galactopyranoside (100 µg/mL). The 2-chlorophenol peak was easily identifiable on the chromatogram and it is well separated from the phenol peak (t_R = 16 min) (Figure 6.4).

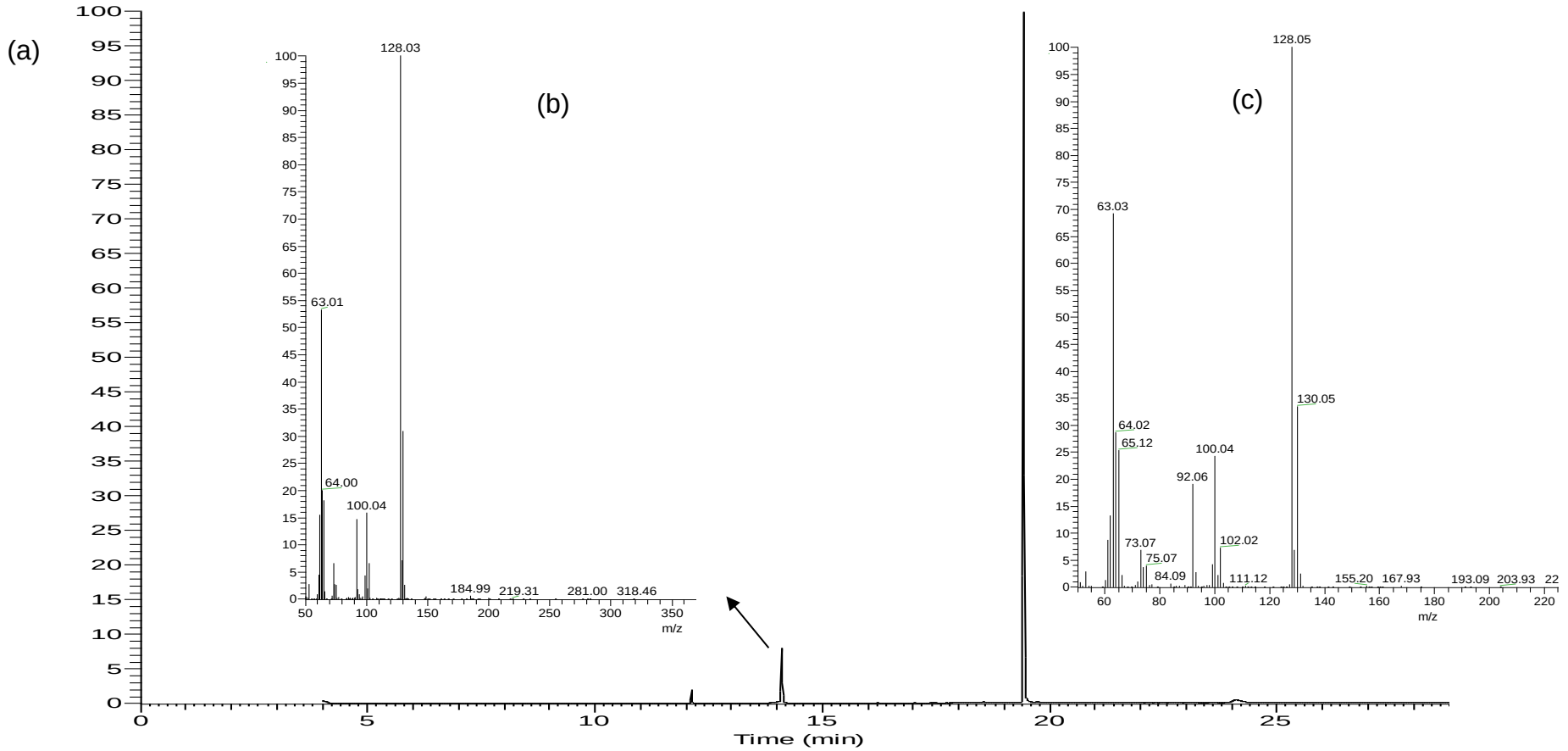


Figure 6.3 is the 2-chlorophenol peak liberated by *S. stanley* inoculated in RVS with 100 µg/mL of 2-chlorophenyl octanoate using a polar GC column and a polar SPME fiber (t_R = 12.1 min is NMP, t_R = 14.1 min is 2-chlorophenol, and t_R = 19.5 min is the substrate 2-chlorophenyl octanoate (b) is the mass spectrum of 2-chlorophenol generated by *S. stanley* through C8 esterase activity inoculated in RVS, and (c) is the mass spectrum of standard 2-chlorophenol

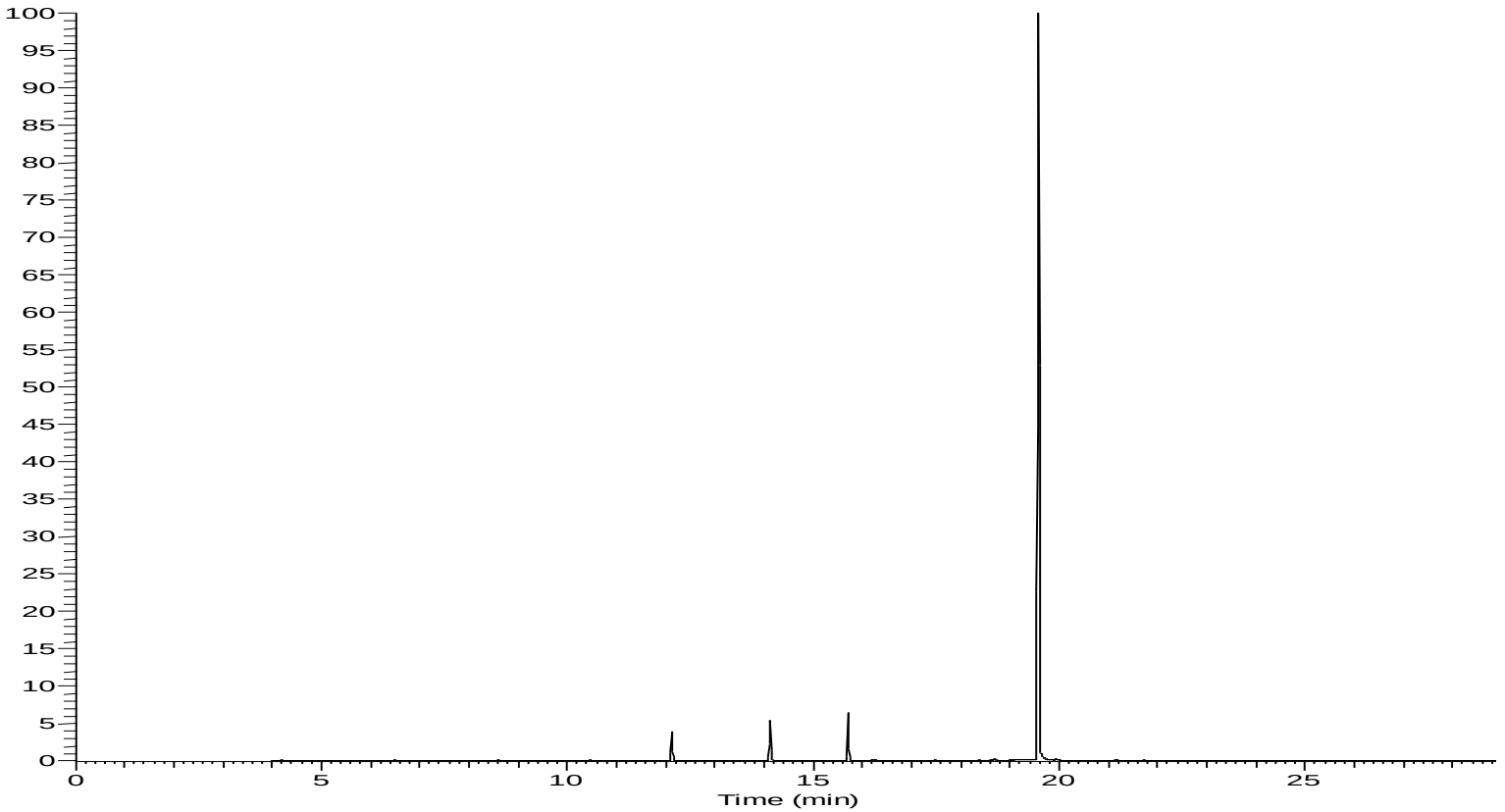


Figure 6.4 VOC profile liberated by *S. stanley* inoculated with phenyl α-D-galactopyranoside and 2-chlorophenyl octanoate t_R = 12.1 min is NMP, t_R = 14.1 min is 2-chlorophenol, t_R = 15.7 min is phenol, t_R = 19.5 min is 2-chlorophenyl octanoat

The VOCs 2-chlorophenol and phenol were produced with mean amounts produced ± one standard deviation being 1.4 ± 0.2 µg/mL and 29.1 ± 3.2 µg/mL, respectively.

6.4.1 Application of optimised method to food samples

Food samples tested include 4 types of milk; whole milk, semi skimmed milk, goat milk, full cream milk, 6 types of cheese; cheddar cheese, goat milk cheese Bassett stilton cheese, Claxton blue cheese, Roquefort cheese, brie blue cheese, 4 types of chicken; skin-less breast chicken, skin-on breast chicken, chicken wings chicken thigh and drumstick and 3 types of egg samples; free range eggs, caged hen eggs and organic eggs. The liberated VOCs from un-spiked and spiked samples detected in the headspace of the samples by GC/MS and the bacteria isolated were identified by MALDI/TOF-MS.

The identification experimental details of the isolates can be found in Section 3.8.2 and Section 3.11.3. The plating medium ABC is selective for *Salmonella*, and the CLED is a non-selective medium that supports the growth of pathogens and contaminants but inhibits the swarming of *Proteus* for good isolation. All food sample tested were found to be *Salmonella*-negative. Regarding to VOCs detected, un-spiked samples gave positive signal response indicating the presence of enzyme activity linked to bacteria. Spiked samples should give positive signal response for C-8 esterase and α- galactosidase, and negative response for PYRase. All the VOCs liberated were detected with high sensitivity and were quantifiable and above the quantification limits. The analysis results of each food type will be discussed individually in the following.

6.4.1.1 Milk samples

The liberated VOCs and the isolated pathogenic are detailed in Tables 6.7-6.10. The VOC profiles of spiked and un-spiked milk samples are present in Figure 6.5. All pasteurized milk samples tested were *Salmonella* free, according to MALDI-TOF results; also, there is no false positive results detected in milk samples as no *Salmonella* enzyme activities detected. Therefore, this detection method successfully eliminates detection of false positive results in milk samples.

In pure whole milk samples and semi skimmed milk samples, the only VOC detected is 2-chlorophenol and the only bacteria isolated on CLED medium are the Gram-positive *Streptococcus salivarius.* The detected C-8 esterase activity is up to the isolated *Streptococcus salivarius*. These bacteria are well known to have this kind of activity (Kalantzopoulos *et al*., 1990). In the spiked samples of whole milk and semi skimmed milk, the detection of 2-chlorophenol and phenol as well as the production of green colonies on the *Salmonella* selective ABC medium were as expected due to the presence of *S. stanley*. No VOCs were detected and no bacteria isolated in un-spiked goat milk samples; in the spiked samples the detected VOCs were as expected which made the results significant. Table 6.9 shows that the Gram negative *Acinetobacter sp*. and the Gram positive *Enterococcus faecalis* were detected in full cream milk samples with detection of 2-chlorophenol, phenol and 3-fluoroaniline. *Acinetobacter spp.* were isolated in *Salmonella* ABC medium as black colonies (Perry *et al*., 1999). *Acinetobacter spp.* have a positive signal for C-8 esterase activity (Freydiere and Gille, 1991) while the 3-fluoroaniline is due to the presence of pyrrolidnyl peptidase in *Enterococcus faecalis* (Gordon *et al*., 1988). However, in the literature there is no evidence for the α-galactosidas activity in *Enterococcus faecalis* and *Acinetobacter spp..*

Table 6.7 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) whole milk samples detected by HS-SPME GC/MS and MALDI/TOF

Whole milk (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	0.40	2.0	*Streptococcus salivarius*	NG
		2	0.54	2.1	*Streptococcus salivarius*	NG
		3	0.54	1.8	*Streptococcus salivarius*	NG
α- Galactosidase	Phenol (µg /mL)	1	ND	4.8	*Streptococcus salivarius*	NG
		2	ND	8.5	*Streptococcus salivarius*	NG
		3	ND	12.5	*Streptococcus salivarius*	NG
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	*Streptococcus salivarius*	NG
		2	ND	ND	*Streptococcus salivarius*	NG
		3	ND	ND	*Streptococcus salivarius*	NG

ND =not detected, NG = no growth

Table 6.8 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) semi skimmed milk samples detected by HS-SPME GC/MS and MALDI/TOF

Semi-skimmed (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	0.79	3.70	*Streptococcus salivarius*	NG
		2	0.73	2.75	*Streptococcus salivarius*	NG
		3	0.77	4.01	*Streptococcus salivarius*	NG
α- Galactosidase	Phenol (µg /mL)	1	ND	9.90	*Streptococcus salivarius*	NG
		2	ND	6.74	*Streptococcus salivarius*	NG
		3	ND	8.20	*Streptococcus salivarius*	NG
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	*Streptococcus salivarius*	NG
		2	ND	ND	*Streptococcus salivarius*	NG
		3	ND	ND	*Streptococcus salivarius*	NG

ND = not detected, NG = no growth

Table 6.9 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) goat milk samples detected by HS-SPME GC/MS and MALDI/TOF

Goat milk (24 hours)						
Enzyme	**Label**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	ND	0.95	NG	NG
		2	ND	3.40	NG	NG
		3	ND	3.20	NG	NG
α- Galactosidase	Phenol (µg /mL)	1	ND	17.4	NG	NG
		2	ND	24.9	NG	NG
		3	ND	22.6	NG	NG
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	NG	NG
		2	ND	ND	NG	NG
		3	ND	ND	NG	NG

ND = not detected, NG = no growth

Table 6.10 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) full cream milk samples detected by HS-SPME GC/MS and MALDI/TOF

Full-cream milk (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	1.34	1.19	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*
		2	1.42	3.63	*Enterococcus faecalis*	*NG*
		3	1.91	1.98	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*
α- Galactosidase	Phenol (µg /mL)	1	15.2	18.6	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp*
		2	1.80	5.11	*Enterococcus faecalis*	*NG*
		3	1.70	5.40	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*
PYRase	3-Fluoroaniline (µg /mL)	1	1.20	1.40	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*
		2	0.97	1.40	*Enterococcus faecalis*	*NG*
		3	1.00	1.30	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*

NG = no growth

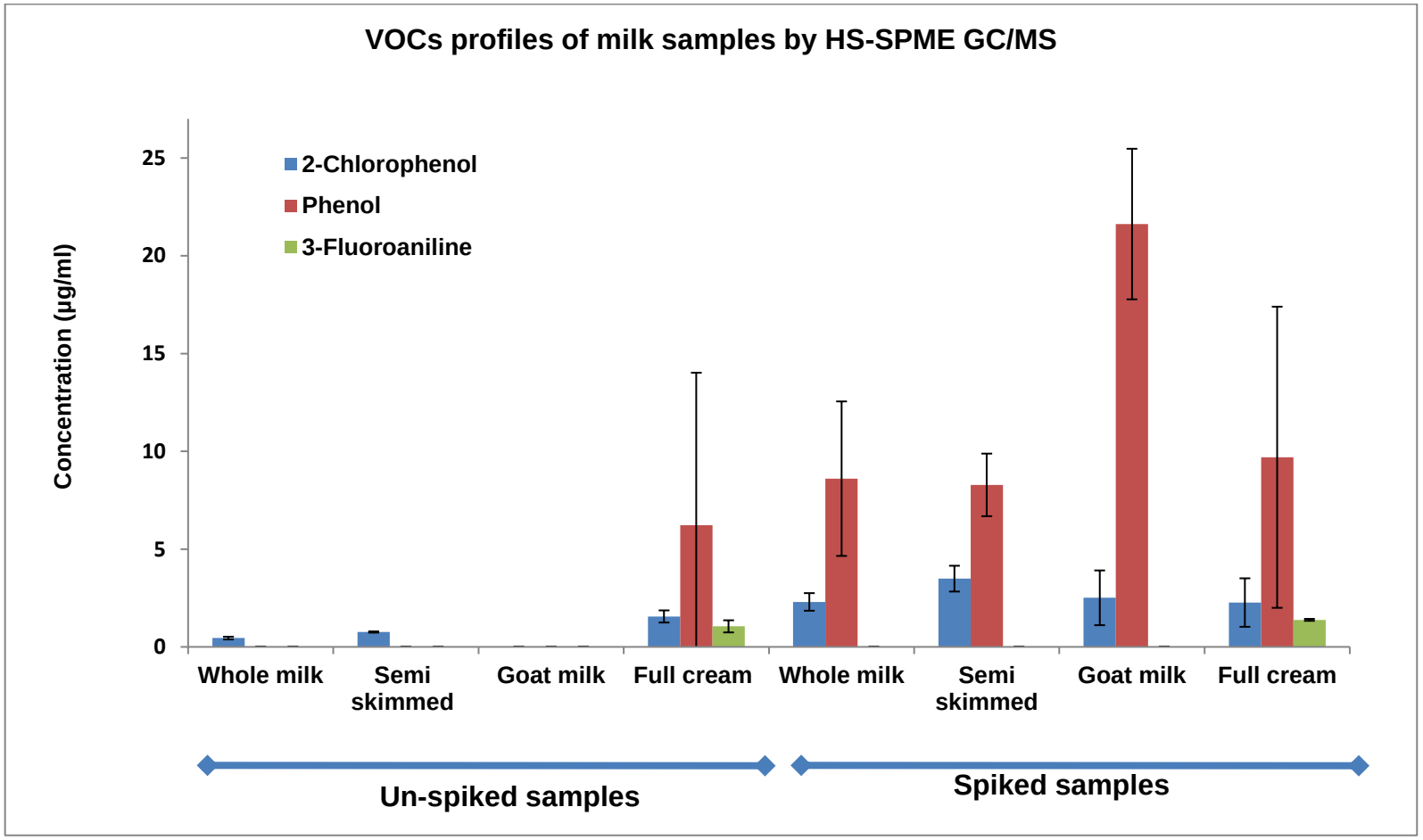


Figure 6.5 Figure 6.5 VOC profiles of milk samples by HS-SPME GC/MS

Therefore, the detected phenol in full cream milk samples needs more investigation by studding the enzyme activity (α-galactosidase) in the *isolated bacteria* (Chapter 7).

Most bacteria considered natural to the milk sources (Murphy and Boor, 2000), however, pathogenic bacteria enter milk from a variety of sources such as unsanitary handling even after the completion of the pasteurization process. *Streptococcus salivarius* is naturally found in raw milk (Quigley *et al*., 2013) and it is one of the microorganisms facilitating dairy fermentations. In addition, *Acinetobacter sp*. and *Enterococcus faecalis* had previously been associated with raw milk (Quigley *et al*., 2013). This implies the occurrence of poor sterility or improper pasteurization. Typically, pasteurization is effective in reducing microbial risks, but some bacteria survive pasteurization; these are called thermoduric bacteria (Hileman, 1940). Thermoduric bacteria in milk are most commonly associated with some contamination source. The various species of the genus *Streptococcus* and *Enterococcus* are described as heat resistant species (Marth and Steele, 2001) and *Enterococcus faecalis* observed exhibiting the greatest heat resistance (Mcauley *et al*., 2012). This fact explains detection of *Streptococcus salivarius*, *Enterococcus faecalis* and *Acinetobacter spp*. in pasteurized milk samples. These pathogens could be present in very low level in the samples and the long incubation period (16-20 h at 37 ºC), that often required as pre enrichment step increase their level in the samples.

6.4.1.2 Cheese samples

This Section contains results and discussion for the analysis performed on six cheese types specifically cheddar cheese, Bassett stilton cheese, goat milk

cheese, Claxton blue cheese, brie cheese and Roquefort cheese. The VOC profiles of the cheese samples are set out in Tables 6.11 - 6.16 and Figure 6.6.

No VOCs detected in any blank samples. And by identifying the isolated bacteria in the cheese samples using MALDI/TOF it is apparent from these results that tested cheeses are *Salmonella* free. The VOCs 2-chlorophenol, phenol and 3-fluoroaniline were detected in all un-spiked cheese samples indicating presence of some bacteria in the cheeses samples tested and their enzymes hydrolysed the inoculated substrates.

Results from identification of the isolates show that the Gram-positive *Enterococcus faecalis* were the only isolates in the three replicate samples of goat cheese. These bacteria have been previously isolated from different cheese types (Baumgartner *et al*., 2001). According to Bulajić and Mijačević (2004) *Enterococcus faecalis* have been proposed as part of defined starter cultures for different cheeses and has been reported to accelerate maturation and to improve organoleptic characteristics of cheeses, specially goat milk cheese (Tzanetakis *et al*., 1995). This fact explains the isolation of *Enterococcus faecalis* from the tested goat milk cheese. In goat cheese samples the three VOCs signals were detected with quantifiable amount (Table 6.10). As mentioned in the previous Section, *Enterococcus faecalis* have positive PYRase activity and have been previously reported by Gordon *et al*., (1988). In reviewing the literature, no data was found on the presence/absence of C-8 esterase and α- galactosidase activity of *Enterococcus faecalis*. Therefore, to explain the detected 2-chlorophenol and phenol investigation on experiment will be carried out testing these enzyme activities of *Enterococcus faecalis* in pure culture (Chapter 7). In the cheddar cheese samples, both the Gram-negative pathogenic *Escherichia coli* and

Proteus vulgaris were isolated on CLED agar. However, on the selective ABC agar, only *Escherichia coli* was able to grow and produced black colonies matching those observed in an earlier study (Perry *et al*., 1999); *Proteus vulgaris* did not isolate using ABC medium as expected. A possible explanation for this result could be the heavy growth of *E. coli* which hidden the colonies of *Proteus vulgaris* in the plate; or the identical look of the colonies of both species. Cheddar cheese is a good substance for the growth of certain species of bacteria due to its low pH, elevated salt concentration and low water activity (Pitt and Hocking, 1997). In a study conducted by Coton *et al*. (2012), it was shown that pathogenic Gram-negative *Escherichia coli* and *Proteus vulgaris* are dairy bacteria associated with French cheeses. Another support for our finding is the study by Torkar and Teger (2006) who reported the presence of pathogenic *Escherichia coli* and *Proteus vulgaris* in salted and non-salted cheese samples. There are many different types of *Escherichia coli* and *Proteus vulgaris* some of them are harmful and some are not harmful to humans.

Escherichia coli is more dangerous to human than *Proteus vulgaris* as more than 80% of humans urinary tract infections are due to the bacterium *E. coli* (Phage Therapy Center, 2016). The most infamous harmful strain of *E. coli* is O157:H7. *Escherichia coli* 0157:H7, has been found in low as well as high moisture cheese as a result of poor pasteurization (Frye and Donnell, 2005). *E. coli* outbreaks associated with consumption of different varieties of dairy products have been reported in several countries (Kwenda *et al*., 2014). The first adequately documented occurrence of enteropathogenic *E. coli* foodborne disease in the U.S.A emerged during the 1970s with first serious outbreak traced to imported French cheese (Marier *et al*., 1973) and more recently, a child dies in

E. coli infection outbreak linked to blue cheese in Scotland (child dies in *E. coli* bug outbreak linked to blue cheese, 2016).

In cheddar cheese sample only detected phenol can be explained by the fact that some strains of *Escherichia coli* species are accountable for α-galactosidase activity (Kämpfer *et al*., 1991). The other two detected VOCs could not be explained as *Escherichia coli* species are known to be negative for C-8 esterase (Dealler *et al*.1992) and also negative for PYRase (Freydiere and Gille, 1991). In addition, *Proteus vulgaris* are reported to be negative for C-8 esterase (Freydiere and Gille, 1991), α-galactosidase (Kämpfer *et al*., 1991) and PYRase (Inoue *et al*., 1996). Therefore, the detected PYRase activity and C-8 esterase activity in the isolated bacteria need more investigation to find a logical source of 2-chlorophenol and 3-fluoroanaline. With successive isolation and identification in CLED agar and ABC agar plates, the Gram negative *Acinetobacter spp*. was detected in Bassett stilton cheese samples. Therefore, *Acinetobacter sp* are responsible for the detected 2-chlorophenol signal that clarify the incidence of C-8 esterase activity in these bacteria (Freydiere and Gille., 1991). The Gram positive *Enterococcus spp.* and *Enterococcus faecalis* were also detected in Bassett stilton cheese samples using CLED medium. The signal of 3-fluoroaniline observed is due to presence of pyrrolidonyl peptidase in *Enterococcus faecalis* and *Enterococcus sp* (Gordon *et al*., 1988) and may be *Acinetobacter sp*. contribute and liberate 3-fluoroanaline as PYRase activity of these bacteria is variable and dependant on species (Bomicino *et al*. 2007). Phenol was also detected and quantified in Bassett stilton cheese, and because α-galactosidase activity is unknown for, *Acinetobacter sp*., *Enterococcus faecalis* and *Enterococcus sp.* this result therefore needs more investigation by testing in pure cultures of these isolates.

In Claxton blue cheese, *Acinetobacter spp*. was the only type of bacteria identified on CLED medium and did not grow on ABC agar plates, this could be due to the slow growth on the *Salmonella* selective medium and/or the low level of *Acinetobacter spp.* on the sample. *Acinetobacter spp*. as mentioned above have C-8 esterase activity and not α-galactosidase and PYRase activity. This explains only the detected 2-chlorophenol while the other two VOCs (phenol and 3-flouroaniline) could not provide clear evidence from which bacteria they were liberated.

Outbreaks of *Salmonella* due to cheese made from unpasteurized milk were often reported (Gould *et al*., 2014). Therefore, detection of *Salmonella* in cheese made from unpasteurised milk was carried out. Roquefort cheese and brie cheese are blue cheeses made from unpasteurized milk. Samples of these cheeses were analysed and no *Salmonella* detected in these cheeses. It can be seen from the Table 6.14 that *Proteus haauseri* were the only bacteria isolated from Roquefort cheese samples using CLED agar plates. While the bacteria isolated from brie cheese samples are more varied; they include *Serratia marcescens* which was isolated on CLED medium and *Enterococcus faecalis* and *Enterobacter cloacae* isolated using blood agar plates. It has been reported (Bulajić and Mijačević, 2004) that the predominant microorganism in some European cheeses is *Enterococcus faecalis* and this is supported in these finding. In French cheese samples Coton *et al*., (2011) isolated and identified some Gram negative bacteria belonging to the *Enterobacteriaceae* family at the species level and these include; *Serratia*, *Proteus* and *Enterobacter* species.

Table 6.11 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) goat cheese samples detected by HS-SPME GC/MS and MALDI/TOF

Goat cheese (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	1.30	2.60	*Enterococcus faecalis*	NG
		2	2.10	1.80	*Enterococcus faecalis*	NG
		3	1.60	2.00	*Enterococcus faecalis*	NG
α- Galactosidase	Phenol (µg /mL)	1	3.10	5.85	*Enterococcus faecalis*	NG
		2	6.60	4.70	*Enterococcus faecalis*	NG
		3	7.10	5.70	*Enterococcus faecalis*	NG
PYRase	3-Fluoroaniline (µg /mL)	1	1.30	1.40	*Enterococcus faecalis*	NG
		2	1.12	1.11	*Enterococcus faecalis*	NG
		3	1.10	1.11	*Enterococcus faecalis*	NG

NG = no growth

Table 6.12 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) cheddar cheese samples detected by HS-SPME GC/MS and MALDI/TOF

Cheddar cheese (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	0.31	0.57	*Escherichia coli*	*Escherichia coli*
		2	0.46	0.72	*Escherichia coli*	*Escherichia coli*
		3	0.34	0.44	*Escherichia coli, Proteus vulgaris*	*Escherichia coli*
α- Galactosidase	Phenol (µg /mL)	1	9.70	14.6	*Escherichia coli*	*Escherichia coli*
		2	4.20	21.0	*Escherichia coli*	*Escherichia coli*
		3	4.00	20.6	*Escherichia coli, Proteus vulgaris*	*Escherichia coli*
PYRase	3-Fluoroaniline (µg /mL)	1	1.70	1.30	*Escherichia coli*	*Escherichia coli*
		2	1.40	1.20	*Escherichia coli*	*Escherichia coli*
		3	1.14	1.10	*Escherichia coli, Proteus vulgaris*	*Escherichia coli*

Table 6.13 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) bassett stilton cheese samples detected by HS-SPME GC/MS and MALDI/TOF

Bassett stilton cheese (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	1.15	2.96	*Acinetobacter sp., Enterococcus sp.*	*Acinetobacter sp.*
		2	1.00	2.83	*Enterococcus sp.*	*NG*
		3	5.97	7.20	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*
α- Galactosidase	Phenol (µg /mL)	1	19.8	34.2	*Acinetobacter sp., Enterococcus sp.*	*Acinetobacter sp.*
		2	20.0	29.0	*Enterococcus sp.*	*NG*
		3	17.2	23.9	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*
PYRase	3-Fluoroaniline (µg /mL)	1	2.80	2.97	*Acinetobacter sp., Enterococcus sp.*	*Acinetobacter sp.*
		2	1.73	1.82	*Enterococcus sp.*	*NG*
		3	1.50	1.42	*Acinetobacter sp., Enterococcus faecalis*	*Acinetobacter sp.*

Table 6.14 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) claxton blue cheese samples detected by HS-SPME GC/MS and MALDI/TOF

Claxton blue cheese (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	0.98	2.44	*Acinetobacter sp.*	NG
		2	0.93	3.40	NG	NG
		3	2.39	4.20	NG	NG
α- Galactosidase	Phenol (µg /mL)	1	33.3	37.8	*Acinetobacter sp.*	NG
		2	26.1	31.2	NG	NG
		3	32.6	37.3	NG	NG
PYRase	3-Fluoroaniline (µg /mL)	1	1.43	1.62	*Acinetobacter sp.*	NG
		2	1.57	1.79	NG	NG
		3	2.40	2.30	NG	NG

NG = no growth

Table 6.15 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) Roquefort cheese samples detected by HS-SPME GC/MS and MALDI/TOF

Roquefort cheese (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (blood agar))**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	0.92	0.66	*NG*	*NG*
		2	0.43	0.79	*NG*	*NG*
		3	0.46	0.68	*Proteus haauseri*	*Proteus haauseri*
α- Galactosidase	Phenol (µg /mL)	1	17.6	37.2	*NG*	*NG*
		2	12.0	38.5	*NG*	*NG*
		3	15.2	34.3	*Proteus haauseri*	*Proteus haauseri*
PYRase	3-Fluoroaniline (µg /mL)	1	1.22	2.80	*NG*	*NG*
		2	1.20	2.70	*NG*	*NG*
		3	1.10	2.60	*Proteus haauseri*	*Proteus haauseri*

NG = no growth

Table 6.16 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) brie blue cheese detected by HS-SPME GC/MS and MALDI/TOF

Brie cheese (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (blood agar)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	1.40	1.50	*NG*	*Enterobacter cloacae*
		2	1.40	2.40	NG	NG
		3	1.50	1.40	*Serratia marcescens*	*Enterococcus faecalis*
α- Galactosidase	Phenol (µg /mL)	1	4.10	31.2	*NG*	*Enterobacter cloacae*
		2	4.90	28.2	NG	NG
		3	4.30	31.3	*Serratia marcescens*	*Enterococcus faecalis*
PYRase	3-Fluoroaniline (µg /mL)	1	2.90	4.40	*NG*	*Enterobacter cloacae*
		2	5.00	3.80	NG	NG
		3	3.80	5.30	*Serratia marcescens*	*Enterococcus faecalis*

NG = no growth

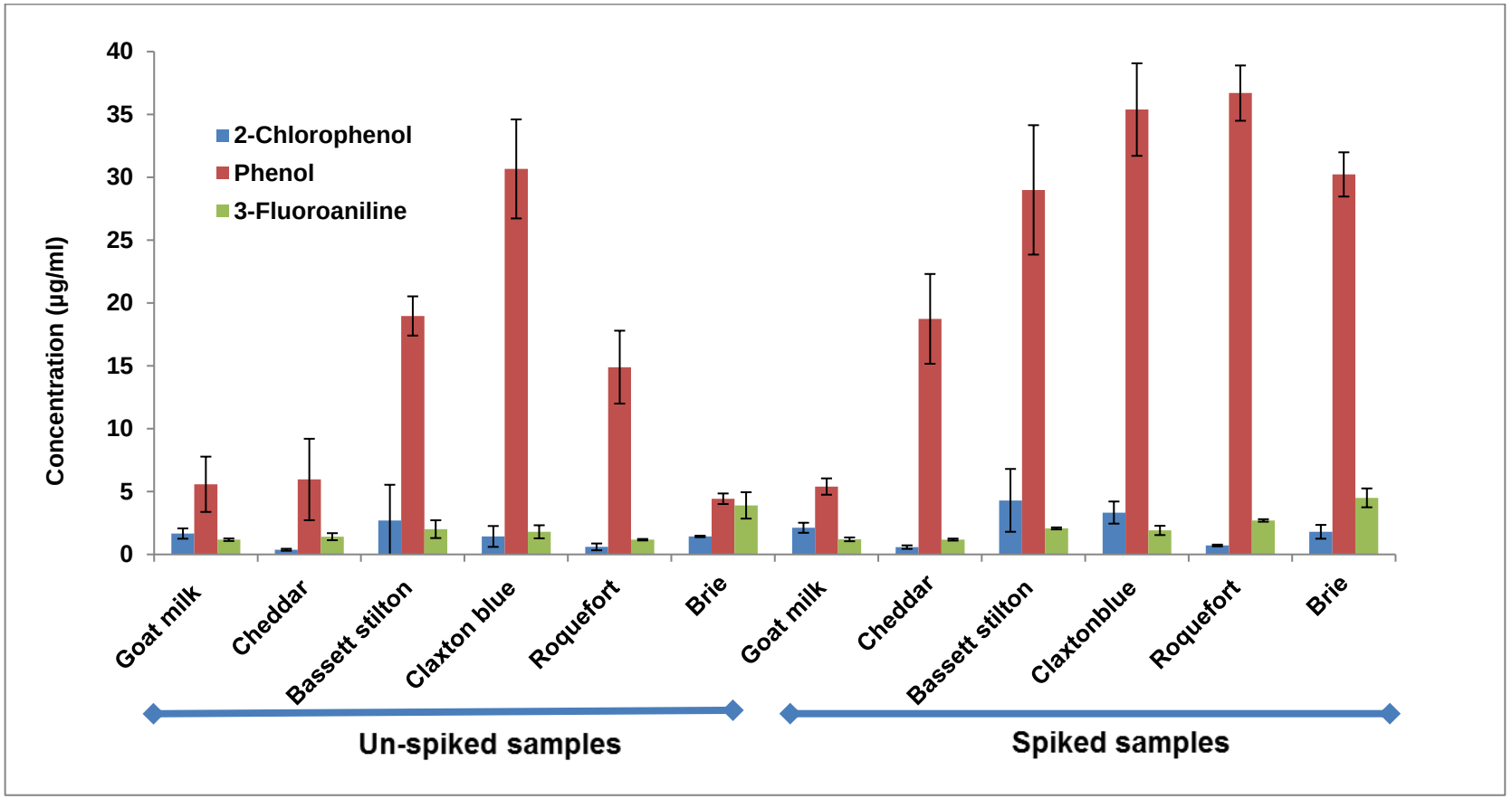


Figure 6.6 VOC profiles for cheese samples by HS SPME GC/MS

The enzyme activities of *Proteus haauseri* were reported to be negative for all enzymes tested; C-8 esterase (Freydiere and Gille, 1991), α- galactosidase Kämpfer *et al*., 1991) and PYRase (Inoue *et al*.1996). The C-8 esterase activity was reported to be negative to each of *Enterobacter cloacae* (Cooke *et al*., 1999) and *Serratia marcescens* (Freydiere and Gille, 1991; Cooke *et al*., 1999) and unknown to *Enterococcus faecalis*. Therefore, the detected C-8 esterase activity in Roquefort and brie cheese samples could not be explained. In the same way the detected α- galactosidase activity in these isolates needs more investigation in pure culture as it is reported to be negative to *Serratia marcescens* (Freydiere and Gille, 1991) and unknown to both *Enterococcus faecalis and Enterobacter cloacae.*

6.4.1.3 Raw chicken samples

The experiment outlined in this Section seeks to identify *Salmonella* in chicken samples. Four different types of chicken samples (skinless and skin-on breast fillets, chicken wings and chicken thighs & drumsticks thighs) were analyzed in 2 replicate samples utilizing enzyme substrates liberating VOCs. The enzyme substrates were hydrolyzed and the exogenous VOCs were detected and quantified. The results of the detected VOCs are shown in Figure 6.4. And the results of quantitative analysis of VOCs and the identified isolates detected in chicken samples are presented in Tables 6. 17 - 6.20. From the data in Figure 6.4, it is apparent that the three substrates are hydrolyzed in all chicken samples and the three VOCs detected with the exception of thigh and drumstick samples, where 3-fluoroanaline did not detected. This suggested the presence of bacteria that are positive for the targeted enzymes in the utilized approach which *Salmonella* could be one of them as phenol and 2-chlorophenol were detected. The pyrrolidonyl peptidase (PYRase) activity is expressed by some other bacteria

but not by *Salmonella*. However, what stands out in Tables 6.16 - 6.19 through identification of the isolates prove that all chicken samples are *Salmonella* free, and all detected enzymatic activities are expressed to the identified bacteria.

In skinless chicken breast samples Gram-negative *Pseudomonas aeruginosa* was isolated on CLED agar plates. While *Escherichia coli* sp. was identified after growth on both CLED agar plates and on selective ABC medium. These bacteria have been previously found in chicken samples. *Pseudomonas species* have been isolated and identified in chilled chicken samples (Arnaut-Rollier *et al*., 1999); while Zhao *et al*. (2001) reported the presence of *Escherichia coli* in raw chicken samples. The detected phenol in skinless breast chicken samples is due to *Escherichia coli* species as they are reported to perform α-galactosidase activity (Kämpfer *et al*., 1991) while the VOCs 2-chloropenol and 3-fluoroanaline are liberated by *Pseudomonas aeruginosa* which is known to express positive activity to both C-8 esterase (Freydiere and Gille, 1991) and PYRase (Mulczyk & Szewczuk, 1970). *Pseudomonas aeruginosa* is known to be negative to α-galactosidase (Freydiere and Gille, 1991) while *Escherichia coli* have no C-8 esterase activity (Dealler *et al*.1992) and no PYRase activity (Freydiere and Gille, 1991; Kämpfer *et al*., 1991)

In skin-on breast chicken samples the Gram-negative *Klebsiella pneumonia* isolated after growth on CLED agar plates and *Escherichia coli* were isolated on plates of ABC medium. The α-galactosidase activity in both *Klebsiella pneumonia* and *Escherichia coli* was positive and reacts with phenyl α-D-galactopyranoside to liberate the detected phenol (Kämpfer *et al*., 1991). The C-8 esterase activity in *Klebsiella pneumonia* and *Escherichia coli* has been reported previously to be negative (Freydiere and Gille, 1991; Cooke *et al*., 1999; Dealler *et al*.1992). However, 2-chlorophenol was detected. It can thus be

suggested that one of these species if not both must have C-8 esterase activity. This implies further experiments, which take these results into account, will need to be undertaken. 3-Fluoroaniline was detected as an indication of PYRase activity in *Klebsiella pneumonia* which has been reported earlier to be positive (Inoue *et al*. 1996).

In chicken thigh and drumstick samples the Gram-negative bacteria *Morganella morganii were* isolated after growth on CLED agar plates. *M. morganii* have been previously isolated and reported in infected chicken samples (Zhao *et al*., 2012). Two species of *Escherichia coli* were isolated from the chicken thigh and drumstick samples after growth on the selective ABC medium. The two species of *E. coli* hydrolysed phenyl α-D-galactopyranoside and liberated phenol. From the VOC profiles shown in Table 6.18, no signal of 3-fluoroanaline was detected in the samples, however, *Morganella morganii* was reported to be positive for PYRase activity (Inoue *et al*. 1996). The absence of a 3-fluoroanaline signal may be due to the low level of bacteria *Morganella morganii* in the samples and the generated 3-fluoroanaline was lower than the detection limit (0.0049 µg/mL). *Morganella morganii* was reported to be negative for C-8 esterase activity (Freydiere and Gille, 1991), similarly *E. coli* (Dealler *et al*. 1992) however, C-8 esterase activity has been detected in the samples this result indicated one (or both) of the isolated species must produce C-8 esterase enzymes.

The bacteria found in chicken wings samples were the most varied in chicken samples and in all food samples analyzed. These bacteria included *Pseudomonas otitidis*, *Morganella morganii, Aeromonas sp*. and *Proteus mirabilis* which were isolated after growth on CLED agar plates; *Escherichia coli* was the only species isolated after growth on the *Salmonella* selective medium (ABC).

Table 6.17 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU /mL) skin-less breast chicken detected by HS-SPME GC/MS and MALDI/TOF

Skin-less breast chicken (24 hours)					
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED) and (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	5.54	4.60	*Pseudomonas aeruginosa and Escherichia coli*
		2	6.38	5.07	*Pseudomonas aeruginosa* and *Escherichia coli*
α- Galactosidase	Phenol (µg /mL)	1	31.5	33.6	*Pseudomonas aeruginosa and Escherichia coli*
		2	35.7	31.3	*Pseudomonas aeruginosa* and *Escherichia coli*
PYRase	3-Fluoroaniline (µg /mL)	1	1.19	1.08	*Pseudomonas aeruginosa and Escherichia coli*
		2	1.07	0.90	*Pseudomonas aeruginosa* and *Escherichia coli*

Table 6.18 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) Skin-on breast chicken detected by HS-SPME GC/MS and MALDI/TOF

Skin-on breast chicken (24 hours)					
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED) and (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	5.94	5.36	*Klebsiella pneumonia and Escherichia coli*
		2	5.85	5.64	*Klebsiella pneumonia* and *Escherichia coli*
α- Galactosidase	Phenol (µg /mL)	1	26.2	25.3	*Klebsiella pneumonia and Escherichia coli*
		2	30.4	30.8	*Klebsiella pneumonia* and *Escherichia coli*
PYRase	3-Fluoroaniline (µg /mL)	1	3.33	2.63	*Klebsiella pneumonia and Escherichia coli*
		2	3.05	3.49	*Klebsiella pneumonia* and *Escherichia coli*

Table 6.19 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) chicken thigh and drumstick detected by HS-SPME GC/MS and MALDI/TOF

Chicken thigh and drumstick (24 hours)					
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED) and (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	11.4	10.2	*Morganella morganii and Escherichia coli*
		2	9.23	8.00	*Morganella morganii and Escherichia coli*
α- Galactosidase	Phenol (µg /mL)	1	25.2	21.8	*Morganella morganii and Escherichia coli*
		2	27.9	19.1	*Morganella morganii and Escherichia coli*
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	*Morganella morganii and Escherichia coli*
		2	ND	ND	*Morganella morganii and Escherichia coli*

ND = not detected

Table 6.20 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) chicken wings detected by HS-SPME GC/MS and MALDI/TOF

Chicken wings (24 hours)					
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED) and (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	8.31	6.89	*Pseudomonas otitidis, Morganella morganii and Escherichia coli*
		2	7.14	9.30	*Aeromonas sp., Morganella morganii and Escherichia coli*
α- Galactosidase	Phenol (µg /mL)	1	25.7	25.7	*Pseudomonas otitidis, Morganella morganii and Escherichia coli*
		2	25.3	26.3	*Aeromonas sp., Morganella morganii and Escherichia coli*
PYRase	3-Fluoroaniline (µg /mL)	1	2.32	2.33	*Pseudomonas otitidis, Morganella morganii and Escherichia coli*
		2	2.03	2.04	*Aeromonas sp., Morganella morganii and Escherichia coli*

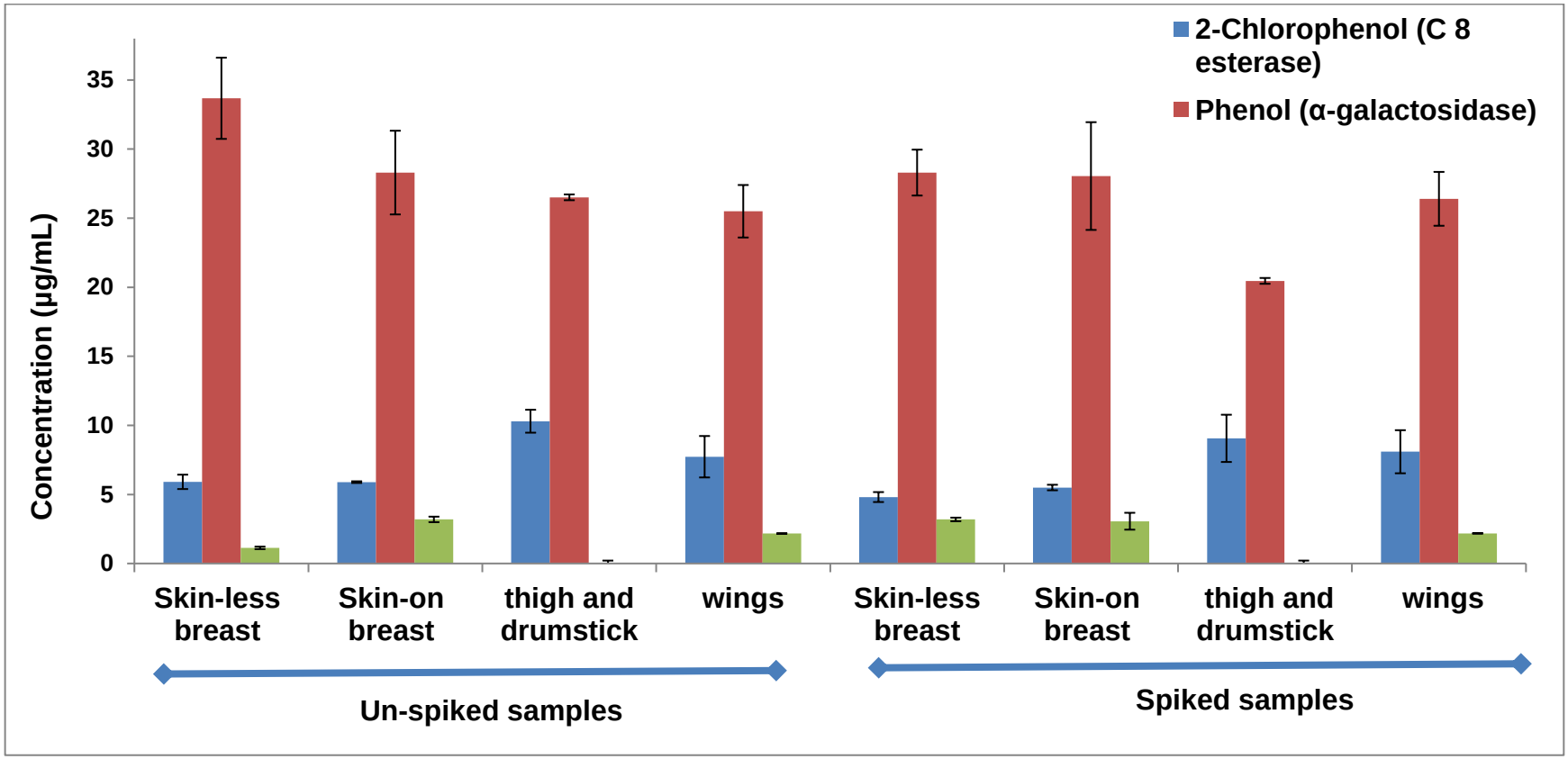


Figure 6.7 VOC profiles for chicken samples by HS SPME GC/MS

The detection of phenol in the samples is due to the hydrolysis of phenyl α-D-galactopyranoside by *Escherichia coli* as they have been reported to have α-galactosidase activity (Kämpfer *et al*., 1991). 3-Fluoroanaline was detected in chicken wings samples verifying the PYRase activity of *pseudomonas otitidis* (Mulczyk & Szewczuk 1970) and *Morganella morganii* (Inoue *et al*. 1996). 2-Chlorophenol has been detected, confirming the presence of the enzyme C-8 esterase in *Aeromonas sp.* as reported by Awan *et al*. (2005) and in *Pseudomonas otitidis,* as reported by Freydiere and Gille (1991).

Most of the species isolated from chicken samples are detected on CLED medium and ABC medium. *E. coli* species were detected as opaque yellow colonies medium *Morganella, Proteus and Pseudomonas* form colourless colonies on ABC medium whereas *E. coli* forms dark black colonies (Perry *et al*., 1999). For some other bacteria for example; *Klebsiella* and *Aeromonas* form black colonies on ABC medium same as *E. coli*.

6.4.1.4 Raw eggs samples

Application of the developed *Salmonella* detection method via detection of exogenous VCs metabolites released by enzymatic hydrolysis in three raw egg types (free range, organic and caged hen eggs) was carried out using the HS-SPME GC/MS method. The enzymatic activities along with the bacteria isolated from the samples are shown in Tables 6.21 - 6.23. The results for VOC profiles of these samples are presented in Figure 6.5. It was found that no *Salmonella* species were detected on both CLED agar and *Salmonella* ABC agar plates. In addition, the α-galactosidase activity which should be positive for *Salmonella* was not detected in all egg samples. Similarly, no PYRase activity was detected in the egg samples and also in the control samples as expected; which confirms the

absence of *Salmonella* in the tested egg samples. The C-8 esterase activity was observed in all samples by detection of 2-chlorophenol indicating the presence of bacteria that express positive C-8 esterase activity.

Gram-negative bacteria *Acinetobacter sp*. was the dominant species in all egg samples and was isolated in both CLED and ABC media. They are well known to produce C-8 esterase (Freydiere and Gille, 1991). Gram-positive *Staphylococcus epidermidis* and Gram-negative *Pseudomonas spp.* were found in free range egg samples as indicated by the liberation of 2-chlorophenol representative of C-8 esterase activity (Freydiere and Gille, 1991). The isolation of *Staphylococcus epidermidis* are in line with those of previous studies (Chaemsanit *et al*., 2015; Jain and Yadav, 2016). *Acinetobacter sp.* and *Pseudomonas spp.* have been reported (Barnes and Corry, 1969) as one of the main spoilage organisms of raw albumen. The Gram-negative *Cuprividus spp*. was found in caged hen egg samples along with *Acinetobacter sp*p. In the literature no information was found about the *Cuprividus spp.* enzymatic activities those targeted in the applied detection method. Therefore, the detected C-8 esterase activity is due to *Acinetobacter spp*. presence in the caged hen eggs samples.

6.5 Summary

This chapter has discussed the results obtained from application of proposed *Salmonella* detection method using enzyme substrates through detection of VOCs. No *Salmonella* contamination was found in analysed milk, cheese, eggs, and chicken samples. The use of 2-chlorophenyl octanoate as the C-8 esterase substrate gave more reproducible and significant results than 2-nitrophenyl octanoate in food samples.

Table 6.21 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) free range eggs detected by HS-SPME GC/MS and MALDI/TOF

Free range eggs (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	3.78	2.97	*Acinetobacter sp.*	*Acinetobacter sp.*
		2	2.54	1.91	*Pseudomonas sp.*	NG
		3	6.12	3.41	*Staphylococcus epidermidis*	*NG*
α- Galactosidase	Phenol (µg /mL)	1	ND	27.9	*Acinetobacter sp.*	*Acinetobacter sp.*
		2	ND	36.6	*Pseudomonas sp.*	NG
		3	ND	33.7	*Staphylococcus epidermidis*	*NG*
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	*Acinetobacter sp.*	*Acinetobacter sp.*
		2	ND	ND	*Pseudomonas sp.*	NG
		3	ND	ND	*Staphylococcus epidermidis*	*NG*

ND = not detected, NG = no growth

Table 6.22 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) caged hen eggs detected by HS-SPME GC/MS and MALDI/TOF

Caged hen eggs (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	3.27	3.82	*Cuprividus sp.*	NG
		2	4.63	4.49	*Acinetobacter sp.*	*Acinetobacter sp.*
		3	1.22	3.01	NG	NG
α- Galactosidase	Phenol (µg /mL)	1	ND	31.2	*Cuprividus sp.*	NG
		2	ND	33.9	*Acinetobacter sp.*	*Acinetobacter sp.*
		3	ND	24.5	NG	NG
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	*Cuprividus sp.*	NG
		2	ND	ND	*Acinetobacter sp.*	*Acinetobacter sp.*
		3	ND	ND	NG	NG

ND = not detected, NG = no growth

Table 6.23 liberated VOCs and isolated pathogens in un-spiked and spiked (*S. stanley* (1-1.5) x 10^4 CFU / mL) organic eggs detected by HS-SPME GC/MS and MALDI/TOF

Organic eggs (24 hours)						
Enzyme	**Label (VOC)**	**Individual results**	**Un-spiked**	**Spiked**	**Isolates on (CLED)**	**Isolates on (ABC)**
C-8 Esterase	2-Chlorophenol (µg /mL)	1	0.26	2.04	NG	NG
		2	3.44	3.95	*Acinetobacter sp.*	NG
		3	2.80	2.99	*Acinetobacter sp.*	*Acinetobacter sp.*
α- Galactosidase	Phenol (µg /mL)	1	ND	28.2	NG	NG
		2	ND	34.0	*Acinetobacter sp.*	NG
		3	ND	29.6	*Acinetobacter sp.*	*Acinetobacter sp.*
PYRase	3-Fluoroaniline (µg /mL)	1	ND	ND	NG	NG
		2	ND	ND	*Acinetobacter sp.*	NG
		3	ND	ND	*Acinetobacter sp.*	*Acinetobacter sp.*

ND = not detected, NG = no growth

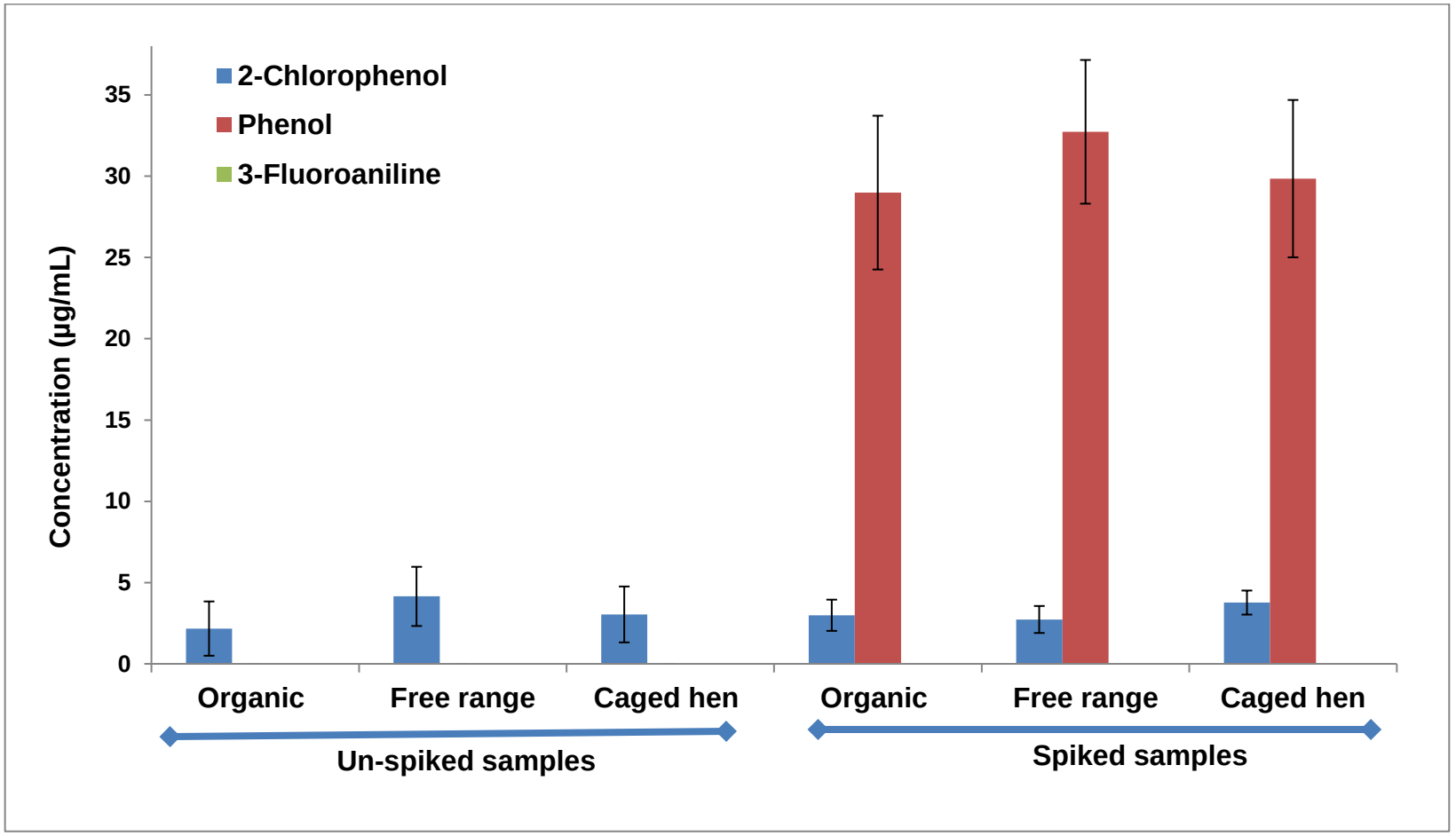


Figure 6.8 VOC profiles for eggs samples by HS SPME GC/MS

This chapter highlights the potential of designing enzyme substrates to liberate exogenous VOCs for *Salmonella* identification. The method was successful as it was able to identify *Salmonella* in spiked samples based on the detection of the expected VOCs.

Food samples are likely to contain high numbers of unknown background flora which interfere with the specificity of the detection method. Having said that, no false positive results were detected in all samples with only one exception detected in chicken thigh and drumstick samples due to the presence of *E. coli* and *Morganella morganii.* This false positive result should not be detected as *Morganella morganii* express PYRase activity which *Salmonella* do not. Not detecting this activity could be due to the low growth level of this bacteria and/or low level of the liberated VOC 3-fluoroanaline. Although *Salmonella* selective broth (RVS) was used in the experiments to inhibit and reduce the growth of Gram positive and some Gram negative bacteria, there are still many bacteria that can be recovered from this broth. To be specific, Gram positive bacteria *Staphylococcus, epidermidis, Streptococcus salivarius,* and *Enterococcus faecalis* and some Gram negative bacteria *Acinetobacter spp., Cuprividus spp., Proteus mirabilis, Escherichia coli,* and *Pseudomonas spp.* were isolated from the tested food samples.

Although the detection and identification of *Salmonella* in food samples through hydrolysis of enzymatic substrates using HS-SPME GC/MS is shown to be rapid and sensitive the presence of other bacteria (pathogenic) in food samples interfere with the specificity of the detection method. Like other projects working with real food samples, we need to overcome the effect of contaminating bacteria on the specificity of the proposed *Salmonella* detection method. It would be interesting to assess the effects of some selective agents or antibiotics, such

as, vancomycin and novobiocin on the specificity and selectivity of the detection method. Adding novobiocin and vancomycin to the *Salmonella* selective RVS broth could inhibit the growth of some Gram-positive bacteria, such as *Enterococcus faecalis, Enterooccus faecium, Staphylococcus epidermidis*, and some Gram negative bacteria, such as, *Proteus spp*.

Unpasteurized milk and unpasteurized raw eggs and raw chicken could contain a very high level of bacteria resistant to a variety of antibiotics (Manie *et al*., 1998) and the presence of such bacteria could cause adversity to the application of the detection method. Therefore, further investigation and experimentation using pasteurized milk and cheese made from pasteurized milk is recommended. The next chapter will discuss the results of experiments conducted on pasteurized milk and cheese made from pasteurized milk incubated in RVS broth with addition of the antibiotics vancomycin (5 mg/L) and novobiocin (10 mg/L).

Chapter 7: Detection of *Salmonella* in milk and cheese samples using antibiotics in the growth media

7.1 Introduction

The results in the previous chapter showed that the detection/identification method of *Salmonella* in food samples through hydrolysis of enzyme substrates using HS-SPME GC/MS is rapid and sensitive. However, because of the presence of other bacteria (pathogenic) the specificity of the method is not as good as required. Since then a decision to add some antibiotics in sufficient quantity to the *Salmonella* selective RVS broth was made to provide effective inhibition of pathogens present in food samples. Raw chicken, unpasteurized milk and unpasteurized raw eggs which are known to contain a very high level of bacteria were rejected; therefore, the less bacteria content food types (pasteurized milk and cheese made from pasteurized milk) were chosen to carry on the experimentation in this chapter. The results of this investigation are discussed below. Experiments investigating the enzymatic activities of representative food isolates were made and have been discussed in detail.

7.2 Vancomycin and novobiocin in *Salmonella* detection method

Novobiocin and vancomycin induces bacterial cell killing however, sufficient concentrations are required for effectively inhibition and from which recovery is not possible. The 5 mg/L of vancomycin and 10 mg/L of novobiocin have been chosen to inhibit bacteria found in the studied milk and cheese samples such as some Gram positive bacteria (e.g. *Enterococcus faecalis, Enterooccus faecium, Enterococcus* spp. and *Staphylococcus epidermidis)* and some Gram negative bacteria, e.g. *Proteus spp*.. This concentration was chosen because it is more effective than other concentrations (Antimicrobial wild type

distributions of microorganisms, no date) based on the minimum inhibitory concentration (MIC), where the lowest concentration of vancomycin that could inhibit the visible growth of such bacteria after overnight incubation is 4 mg/L and similarly the novobiocin, where the MIC is 10 mg/L.

7.3 Milk samples results

The VOC analysis of 3 types of pasteurized milk samples after digestion in BPW and incubation in RVS broth containing vancomycin (5 mg/L) and novobiocin (10 mg/L) was carried out as described in Section 3.11.2. In brief, the antibiotics were added to 9 mL RVS broth containing the three enzyme substrates and 1 mL milk or cheese samples (after digestion in BPW (25 g in 225 mL) for 16-20 h at 37 °C). The samples were incubated for 18-24 h at 37 °C then liberated VOCs were screened using HS-SPME GC/MS. After VOCs analysis, bacteria in the milk and cheese samples were isolated and identified as described in Section 3.11.3.

In the studied milk samples some bacteria were able to grow in RVS broth and resist the effects of vancomycin and novobiocin. These bacteria were able to hydrolyze the inoculated enzyme substrates and liberate VOCs. The VOCs profiles and the isolated pathogens are presented in Tables 7.1 – 7.6. The VOC profiles for individual un-spiked milk samples with antibiotics are shown in Figure 7.1. The VOCs profiles for un-spiked and spiked milk samples with antibiotics are displayed in Figure 7.2.

No *Salmonella* was detected in the milk samples tested. All spiked milk samples showed the expected results in terms of the VOCs detected. In whole milk sample number 1 *Streptococcus salivarius* was isolated on CLED agar plates from un-spiked and spiked samples. The C-8 esterase activity was the only

activity detected in the un-spiked sample as a signal of 2-chlorophenol with a concentration of 1.65 µg/mL. *Streptococcus salivarius* has been reported to have C-8 esterase activity (Kalantzopoulos *et al*., 1990) and negative activity for PYRase (Panosian & Edberg, 1989) which supports the results obtained. In addition, a representative strain, *Streptococcus salivarius* (NCTC 8618) was tested in pure culture (Section 7.6) and showed positive C-8 esterase, negative PYRase and negative α-galactosidase activities, which further supports the obtained results. The C-8 esterase and α-galactosidase activities were detected in the other two whole milk samples. PYRase activity was detected but was below the quantification limit (0.0163 µg/mL). Gram-negative *Enterobacter cloacae* were isolated from both samples. No information was reported regarding the studied enzymatic activities of these species. However, our own work (Section 7.6) reported the positive activity of the 3 substrates tested with *Enterobacter cloacae* (NCTC 11936) in TSB and RVS broth with PYRase activities detected in RVS broth under quantification limit (0.0163 µg/mL) while the concentration of 3-fluoroaniline in TSB was 0.21 µg/mL ± 0.02 µg/mL. This variation on the liberated 3-fluoroanaline is due to nutrients available for the growth of the strain in the formula TSB and RVS.

The results detected in semi skimmed milk samples were similar to that obtained in whole milk samples number 2 and 3. *Enterobacter cloacae* were the only bacteria isolated from semi skimmed milk samples with detection of the C-8 esterase and the α-galactosidase. The PYRase activity was not quantifiable in samples 1 and 2, and in sample number 3 the concentration of 3-fluoroaniline was 0.073 µg/mL. Full cream milk samples showed detection of similar VOCs to previous milk samples.

Table 7.1 liberated VOCs and isolated pathogens in un-spiked whole milk samples incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	1.65	*Streptococcus salivarius*	NG	0.014	0.046
		2	2.56	Enterobacter cloacae	Enterobacter cloacae		
		3	2.97	*Enterobacter cloacae*	Enterobacter cloacae		
α- Galactosidase	Phenol	1	ND	*Streptococcus salivarius*	NG	0.045	0.150
		2	21.60	Enterobacter cloacae	Enterobacter cloacae		
		3	20.90	*Enterobacter cloacae*	Enterobacter cloacae		
PYRase	3-Fluoroaniline	1	ND	*Streptococcus salivarius*	NG	0.005	0.016
		2	NQ	Enterobacter cloacae	Enterobacter cloacae		
		3	NQ	*Enterobacter cloacae*	Enterobacter cloacae		

ND = not detected, NQ = not quantifiable, NG =no growth

Table 7.2 liberated VOCs and isolated pathogens in spiked whole milk samples incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. Stanley* (10^4 CFU/mL) (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	1.35	*Streptococcus salivarius and Salmonella species*	*Salmonella* species	0.0140	0.046
		2	2.95	*Enterobacter cloacae* and *Salmonella* species	*Enterobacter cloacae* and *Salmonella* species		
		3	2.99	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species		
α- Galactosidase	Phenol	1	20.4	*Streptococcus salivarius and Salmonella species*	*Salmonella* species	0.0451	0.1503
		2	20.50	*Enterobacter cloacae* and *Salmonella* species	*Enterobacter cloacae* and *Salmonella* species		
		3	22.10	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species		
PYRase	3-Fluoroaniline	1	ND	*Streptococcus salivarius and Salmonella species*	*Salmonella* species	0.0049	0.0163
		2	NQ	*Enterobacter cloacae* and *Salmonella* species	*Enterobacter cloacae* and *Salmonella* species		
		3	NQ	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species		

ND = not detected, NQ = not quantifiable

Table 7.3 liberated VOCs and isolated pathogens in semi skimmed milk, incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	6.37	*Enterobacter cloacae*	Enterobacter cloacae	0.0140	0.046
		2	1.72	Enterobacter cloacae	Enterobacter cloacae		
		3	7.19	*Enterobacter cloacae*	Enterobacter cloacae		
α- Galactosidase	Phenol	1	32.4	*Enterobacter cloacae*	Enterobacter cloacae	0.0451	0.1503
		2	24.10	Enterobacter cloacae	Enterobacter cloacae		
		3	33.48	*Enterobacter cloacae*	Enterobacter cloacae		
PYRase	3-Fluoroaniline	1	NQ	*Enterobacter cloacae*	Enterobacter cloacae	0.0049	0.0163
		2	NQ	Enterobacter cloacae	Enterobacter cloacae		
		3	0.073	*Enterobacter cloacae*	Enterobacter cloacae		

NQ = not quantifiable

Table 7.4 liberated VOCs and isolated pathogens in spiked semi skimmed milk, incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. Stanley* 10^4 CFU/mL (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	2.88	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species	0.0140	0.046
		2	2.15	*Enterobacter cloacae* and *Salmonella* species	*Enterobacter cloacae* and *Salmonella* species		
		3	1.60	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species		
α- Galactosidase	Phenol	1	29.70	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species	0.0451	0.1503
		2	20.28	*Enterobacter cloacae* and *Salmonella* species	*Enterobacter cloacae* and *Salmonella* species		
		3	31.92	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species		
PYRase	3-Fluoroaniline	1	0.020	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species	0.0049	0.0163
		2	NQ	*Enterobacter cloacae* and *Salmonella* species	*Enterobacter cloacae* and *Salmonella* species		
		3	0.324	*Enterobacter cloacae and Salmonella species*	*Enterobacter cloacae* and *Salmonella* species		

NQ = not quantifiable

Table 7.5 liberated VOCs and isolated pathogens in full cream milk incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C--8 Esterase	2-Chlorophenol	1	2.94	*Enterobacter cloacae* and *Enterococcus faecalis*	NG	0.0140	0.046
		2	2.66	*Enterobacter cloacae, Enterococcus faecalis* and *Streptococcus salivarius*	*Enterobacter cloacae*		
		3	1.60	*Enterococcus faecalis Streptococcus salivarius*	*Enterobacter cloacae*		
α- Galactosidase	Phenol	1	2.00	*Enterobacter cloacae* and *Enterococcus faecalis*	NG	0.0451	0.1503
		2	13.7	*Enterobacter cloacae, Enterococcus faecalis* and *Streptococcus salivarius*	*Enterobacter cloacae*		
		3	1.94	*Enterococcus faecalis Streptococcus salivarius*	*Enterobacter cloacae*		
PYRase	3-Fluoroaniline	1	NQ	*Enterobacter cloacae* and *Enterococcus faecalis*	NG	0.0049	0.0163
		2	NQ	*Enterobacter cloacae, Enterococcus faecalis* and *Streptococcus salivarius*	*Enterobacter cloacae*		
		3	NQ	*Enterococcus faecalis Streptococcus salivarius*	*Enterobacter cloacae*		

NQ = not quantifiable, NG =no growth

Table 7.6 liberated VOCs and isolated pathogens in spiked full cream milk incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. Stanley* 10^4 CFU/mL (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	2.22	*Salmonella species* and *Enterococcus faecalis*	*Salmonella species*	0.0140	0.046
		2	2.54	*Salmonella* species, *Enterobacter cloacae* and *Enterococcus faecalis*	*Salmonella species* and *Enterobacter cloacae*		
		3	3.45	*Salmonella species* and *Enterococcus faecalis*	*Salmonella species*		
α- Galactosidase	Phenol	1	2.60	*Salmonella species* and *Enterococcus faecalis*	*Salmonella species*	0.0451	0.1503
		2	13.42	*Salmonella species*, *Enterobacter cloacae* and *Enterococcus faecalis*	*Salmonella species* and *Enterobacter cloacae*		
		3	2.33	*Salmonella species* and *Enterococcus faecalis*	*Salmonella species*		
PYRase	3-Fluoroaniline	1	0.37	*Salmonella species* and *Enterococcus faecalis*	*Salmonella species*	0.0049	0.0163
		2	NQ	*Salmonella species*, *Enterobacter cloacae* and *Enterococcus faecalis*	*Salmonella species* and *Enterobacter cloacae*		
		3	0.08	*Salmonella species* and *Enterococcus faecalis*	*Salmonella species*		

NQ = not quantifiable

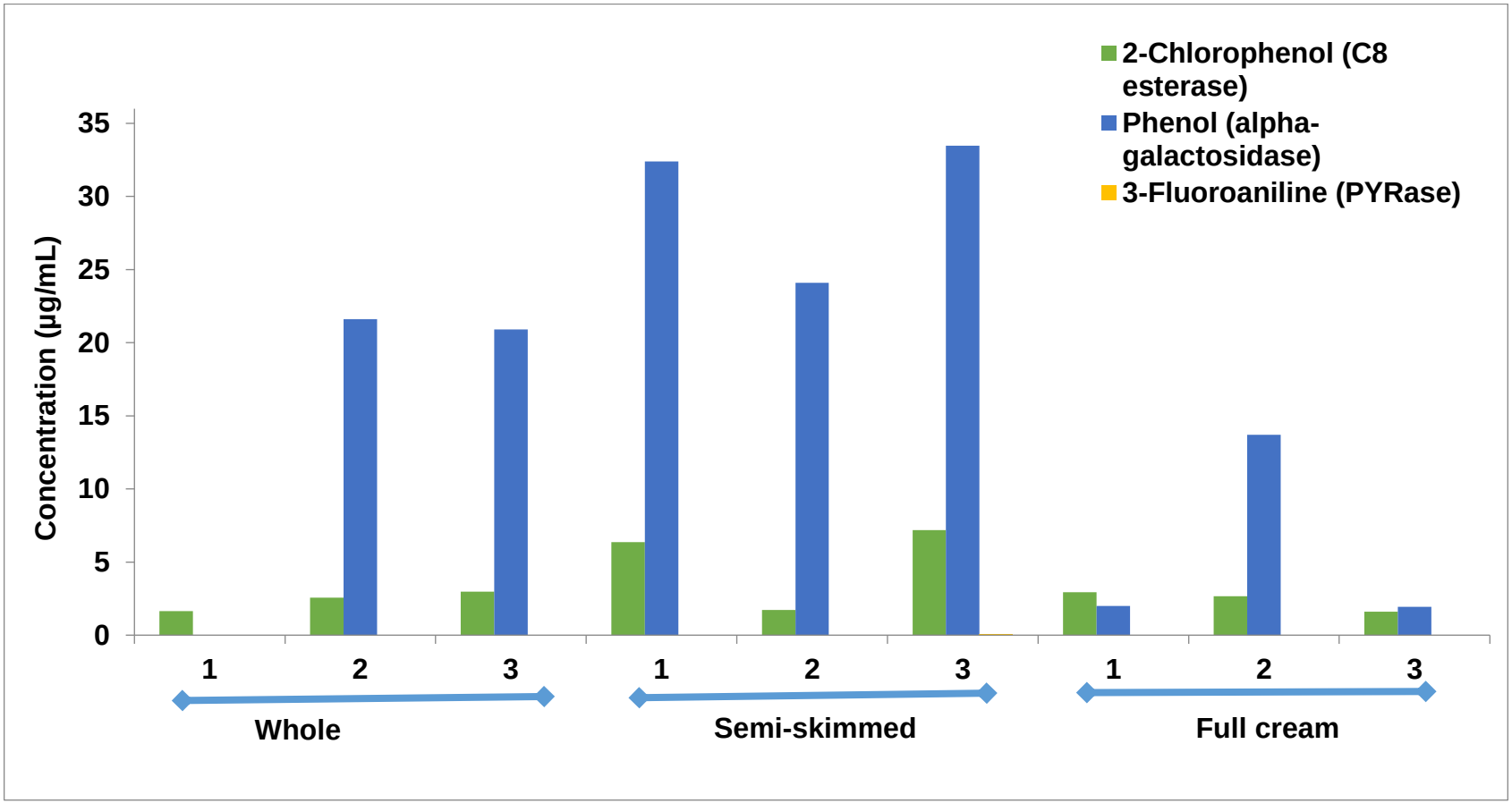


Figure 7.1 VOCs profiles for individual un-spiked milk samples with antibiotics by HS-SPME GC/MS

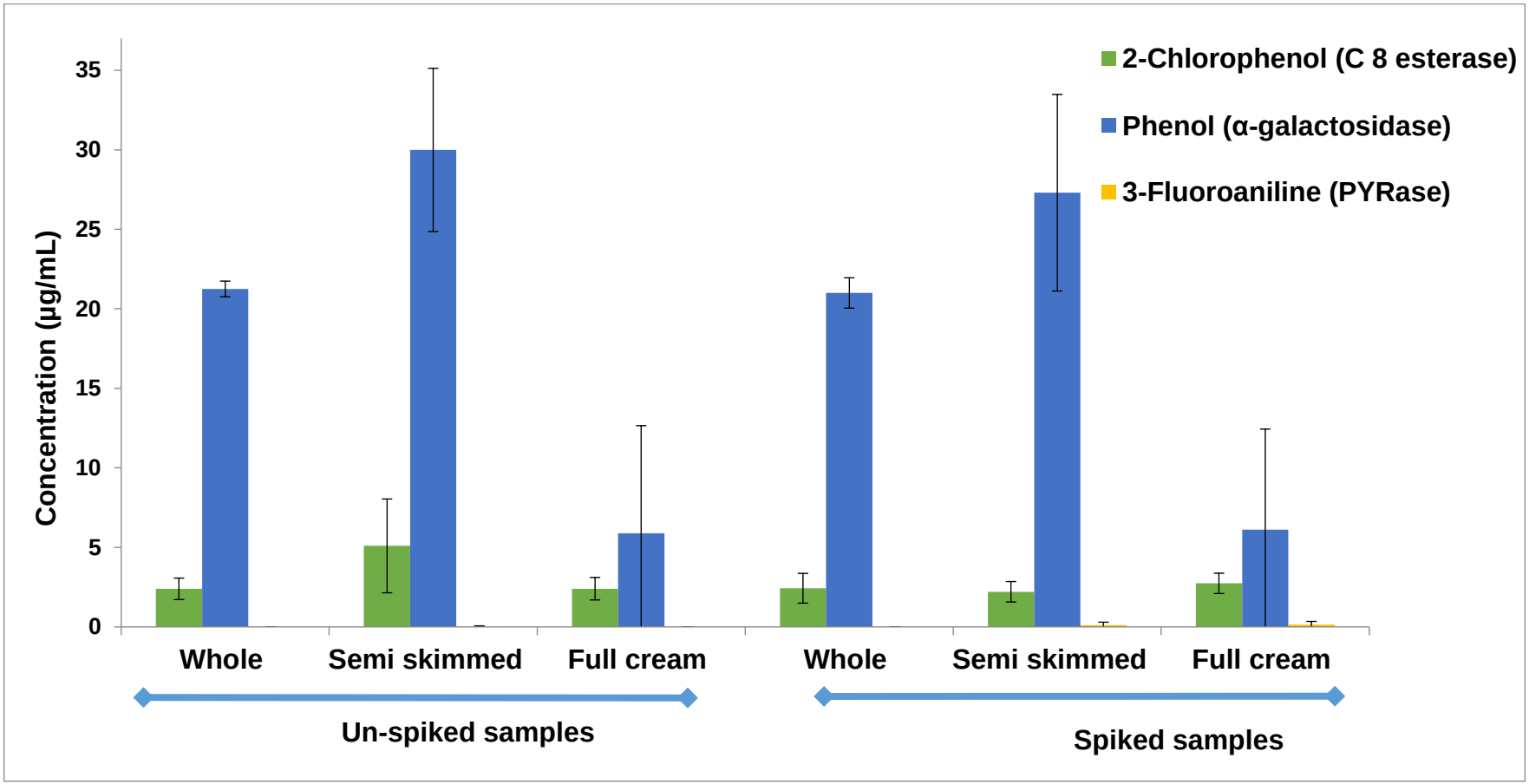


Figure 7.2 VOCs profiles for milk samples with antibiotics by HS-SPME GC/MS

Full cream milk samples showed detection of similar VOCs to previous milk samples. However, the isolated bacteria were more varied in the whole milk and semi skimmed milk as well as in individual full cream milk samples. *Enterobacter cloacae and Enterococcus faecalis* were isolated from full cream sample number 1. *Enterobacter cloacae*, *Enterococcus faecalis* and *Streptococcus salivarius* were isolated from sample number 2 while *Enterococcus faecalis* and *Streptococcus salivarius* were isolated from sample number 3 as has been pointed out in Table 7.5. The detected C-8 esterase activity is due to the presence of the three species *Streptococcus salivarius* (Kalantzopoulos *et al*., 1990) and *Enterobacter cloacae* and *Enterococcus faecalis* (Section 7.6) in full cream milk samples. While the detected α-galactosidase in full cream samples is due to the presence of *Enterobacter cloacae* (Section 7.6) as the other, two isolated species are reported to be negative (Section 7.6). The unquantifiable amount of detected 3-fluoroaniline in all samples is due to presence of *Enterobacter cloacae* (Section 7.6) and *Enterococcus faecalis* (Gordon *et al*., 1988) only, as *Streptococcus salivarius* is known to be PYRase negative (Panosian & Edberg, 1989). There is no interfering result reported in milk samples, however, the detection of false positive results is possible in milk samples as a consequence of the presence of the *Enterobacter cloacae* and *Enterococcus faecalis* when their PYRase activities are not quantifiable.

7.4 Cheese samples results

The VOCs analysis (Section 3.11.2) was carried out to study 4 types of cheese samples in order to screen the samples for *Salmonella* contamination. All cheese samples *were Salmonella* free. However, some resistant bacteria were isolated and are shown along with their liberated VOCs in Table 7.7 to Table

7.14. The VOCs profiles for studied cheese samples are as shown in Figure 7.3 and Figure 7.4. In all cheese samples the inoculated substrates were hydrolyzed as shown by the detection of the 3 VOCs in each sample. This observed result suggests that the targeted enzymes are present in the bacteria found in cheese samples. However, no false positive result was detected as PYRase activity (negative for *Salmonella*) was detected in these samples. The VOCs detected in cheese samples were all quantifiable except the 3-fluoroanaline (PYRase activity) which was unquantifiable and undetectable in some replicates of goat milk cheese and cheddar cheese samples as can be seen in Table 7.7 and Table 7.9, respectively.

Gram-positive *Enterooccus faecium* was the only bacteria isolated from goat cheese samples and was not found in other cheeses. The C-8 esterase and the α-galactosidase activities were detected and quantified in goat cheese samples. The PYRase activity was detected but unquantifiable, in the samples; this activity was previously reported to be positive (Gordon *et al*., 1988). The enzymatic activity investigation of representative strain *Enterococcus faecium* (NCTC 7171) in TSB shown quantifiable mount of 3-fluoroanaline (18.6 ± 0.73 µg/mL) (Own work, Section 7.6). Therefore, the absence and unquantifiable 3-fluoroanaline in goat milk cheese samples (Table 7.7) could be due to the poor growth of *Enterooccus faecium* in RVS. The C-8 esterase activity was proven to be positive when representative strains of *Enterococcus faecium* (NCTC 7171) were tested in TSB and RVS broths (Own work, Section 7.6) and the concentration of detected 2-chlorophenol was 7.39 µg/mL ± 2.4 µg/mL. However, the observed α-galactosidase activity (very small amount compare to normal detected amount in the tested food samples) cannot be due to the presence of *Enterococcus faecium* as the representative strain of *Enterococcus faecium*

(NCTC 7171) was tested and showed negative α-galactosidase activity (Section 7.6). The α-galactosidase activity could be due to the presence of other positive α-galactosidase bacteria that obscured and were not detected due to heavy growth of *Enterococcus faecium* on the plates or could be due to identical look of the colonies of the isolates, which make the differentiation impossible.

In cheddar cheese samples, the 3 VOCs were detected in each individual sample, however, the isolated bacteria varied in the samples (Table 7.9). In cheddar cheese sample number 1 *Enterococcus faecalis* was the only bacteria isolated, while in cheddar cheese number 2*, Enterococcus species,* and *Cronobacter sakazakii* were isolated. In cheddar cheese, sample number 3 *Enterococcus species* was the only isolated bacteria. The enzymatic activities of *Enterococcus faecalis NCTC 775* that represent the isolated *Enterococcus faecalis* tested in RVS and in TSB. IN RVS the only activity detected was the C-8 esterase (2-chlorophenol was 0.6 ± 0.14 µg/mL). Whereas in TSB both C-8 esterase activity (2-chlorophenol was 15.7 ± 1.1 µg/mL and PYRase activity (3-fluoroaniline was 34.5 ± 0.4 µg/mL) were detected (Table 7.14). This strain was negative for α-galactosidase activity when grown in both media (RVS and TSB). Therefore, the detected phenol in cheddar cheese sample 2 (Table 7.9) could be due to *Cronobacter sakazakii* that could not be detected in sample number 1 due to heavy growth of *Enterococcus faecalis,* or to the identical look of the colonies of both species in the culture plates. This interpretation was proven by testing the enzymatic activities (in RVS, and in TSB) of the representative *Cronobacter sakazakii* ATCC 29544; this resulted in detection of the 3 VOCs demonstrating that these bacteria have the targeted enzymes which react with the substrates positively. This finding supported the detected three VOCs in sample number 2 of cheddar cheese.

In Bassett Stilton cheese samples, the inoculated substrates hydrolyzed and the VOCs were detected in all replicates. However, the isolated bacteria in the samples are diverse from one another (Table 7.11). The Gram-negative *Serratia rubidaea* is the dominant species in all Bassett Stilton cheese samples. The enzymatic activities of *Serratia rubidaea* was previously reported to be negative for C-8 esterase (Freydiere and Gille, 1991; Cooke *et al*., 1999) and negative for α-galactosidase (Freydiere and Gille, 1991) while the PYRase activity was reported to be positive (Inoue *et al*., 1996.). The enzymatic activities of *Serratia marcescens* (NCTC 10211) which are closely related to *Serratia rubidaea* were tested (Section 7.6) using RVS and TSB as growth media and found that; the C-8 esterase and the PYRase activities of these bacteria are positive while the α-galactosidase activity is negative. Therefore, one can conclude that, the liberated 2-chlorophenol and 3-fluoroanaline are due to the presence of *Serratia rubidaea* in Bassett Stilton cheese samples. There are, however, other possible explanations to the α-galactosidase activity in the samples, which is the presence of *Enterobacter cloacae* that perform this activity (Section 7.6). As the Gram-negative *Enterobacter cloacae,* NCTC 11936 were tested in RVS and TSB broths and showed positive activities for the three enzymes.

In sample number two, this activity (α-galactosidase) could not be attributed to the isolated bacteria (*Serratia rubidaea* and *Enterococcus faecalis*) in this sample, as they are negative for α-galactosidase. In Bassett Stilton cheese sample number 3 the VOCs were detected and the bacteria isolated were *Serratia rubidaea*, *Enterococcus faecalis* and *Klebsiella oxytoca;* these bacteria were isolated on CLED agar plates and *Providencia rettgeri* were isolated on ABC agar plates.

Table 7.7 liberated VOCs and isolated pathogens in goat milk cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	0.72	*Enterooccus faecium*	NG	0.0140	0.046
		2	2.46	Enterooccus faecium	NG		
		3	1.14	*Enterooccus faecium*	NG		
α- Galactosidase	Phenol	1	ND	*Enterooccus faecium*	NG	0.0451	0.1503
		2	1.37	Enterooccus faecium	NG		
		3	0.93	*Enterooccus faecium*	NG		
PYRase	3-Fluoroaniline	1	ND	*Enterooccus faecium*	NG	0.0049	0.0163
		2	NQ	Enterooccus faecium	NG		
		3	NQ	*Enterooccus faecium*	NG		

ND = not detected, NQ = not quantifiable, NG = no growth

Table 7.8 liberated VOCs and isolated pathogens in spiked goat milk cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. stanley* 10^4 CFU/mL (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	1.99	*Salmonella* and *Enterococcus faecium*	*Salmonella*	0.0140	0.0467
		2	2.50	*Salmonella* and *Enterococcus faecium*	*Salmonella*		
		3	2.16	*Salmonella* and *Enterococcus faecium*	*Salmonella*		
α-Galactosidase	Phenol	1	4.43	*Salmonella and Enterococcus faecium*	*Salmonella*	0.0451	0.1503
		2	2.42	*Salmonella and Enterococcus faecium*	*Salmonella*		
		3	3.85	*Salmonella and Enterococcus faecium*	*Salmonella*		
PYRase	3-Fluoroaniline	1	ND	*Salmonella and Enterococcus faecium*	*Salmonella*	0.0049	0.0163
		2	NQ	*Salmonella and Enterococcus faecium*	*Salmonella*		
		3	0.1	*Salmonella and Enterococcus faecium*	*Salmonella*		

ND = not detected, NQ = not quantifiable

Table 7.9 liberated VOCs and isolated pathogens in cheddar cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	0.13	*Enterococcus faecalis*	NG	0.0140	0.0467
		2	1.38	*Enterococcus species and Cronobacter sakazakii*	Cronobacter sakazakii		
		3	0.28	*Enterococcus species*	NG		
α- Galactosidase	Phenol	1	0.88	*Enterococcus faecalis*	NG	0.0451	0.1503
		2	23.59	Enterococcus species and Cronobacter sakazakii	Cronobacter sakazakii		
		3	ND	*Enterococcus species*	NG		
PYRase	3-Fluoroaniline	1	0.01	*Enterococcus faecalis*	NG	0.0049	0.0163
		2	1.3	Enterococcus species and Cronobacter sakazakii	Cronobacter sakazakii		
		3	ND	*Enterococcus species*	NG		

ND = not detected, NG = no growth

Table 7.10 liberated VOCs and isolated pathogens in spiked cheddar cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. stanley* 10^4 CFU/mL (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	1.56	*Salmonella species*	Salmonella species	0.0140	0.0467
		2	3.03	Salmonella species	*Salmonella species and Cronobacter sakazakii*		
		3	1.90	*Salmonella species*	*Salmonella species*		
α- Galactosidase	Phenol	1	20.16	*Salmonella species*	Salmonella species	0.0451	0.1503
		2	27.60	Salmonella species	*Salmonella species and Cronobacter sakazakii*		
		3	19.16	*Salmonella species*	*Salmonella species*		
PYRase	3-Fluoroaniline	1	0.116	*Salmonella species*	Salmonella species	0.0049	0.0163
		2	2.28	*Salmonella* species	*Salmonella species and Cronobacter sakazakii*		
		3	0.74	*Salmonella species*	*Salmonella species*		

Table 7.11 liberated VOCs and isolated pathogens in Bassett Stilton cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	2.57	*Serratia rubidaea* and *Enterobacter cloacae*	*Serratia rubidaea*	0.0140	0.0467
		2	2.90	*Serratia rubidaea* and *Enterococcus faecalis*	*Serratia rubidaea*		
		3	3.35	*Serratia rubidaea, Enterococcus faecalis* and *Klebsiella oxytoca*	*Serratia rubidaea Providencia rettgeri*		
α- Galactosidase	Phenol	1	19.05	*Serratia rubidaea* and *Enterobacter cloacae*	*Serratia rubidaea*	0.0451	0.1503
		2	8.02	*Serratia rubidaea* and *Enterococcus faecalis*	*Serratia rubidaea*		
		3	15.99	*Serratia rubidaea, Enterococcus faecalis* and *Klebsiella oxytoca*	*Serratia rubidaea Providencia rettgeri*		
PYRase	3-Fluoroaniline	1	5.28	*Serratia rubidaea* and *Enterobacter cloacae*	*Serratia rubidaea*	0.0049	0.0163
		2	3.01	*Serratia rubidaea* and *Enterococcus faecalis*	*Serratia rubidaea*		
		3	2.06	*Serratia rubidaea, Enterococcus faecalis* and *Klebsiella oxytoca*	*Serratia rubidaea Providencia rettgeri*		

Table 7.12 liberated VOCs and isolated pathogens in spiked Bassett Stilton cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. stanley* 10^4 CFU/mL (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	4.39	*Serratia rubidaea,Enterobacter cloacae* and *Salmonella species*	*Serratia rubidaea Salmonella*	0.0140	0.0467
		2	5.16	*Serratia rubidaea, Enterococcus faecalis and Salmonella species*	*Salmonella*		
		3	4.78	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella species*	*Serratia rubidaea Salmonella*		
α- Galactosidase	Phenol	1	21.97	*Serratia rubidaea,Enterobacter cloacae* and *Salmonella species*	*Serratia rubidaea Salmonella*	0.0451	0.1503
		2	28.01	*Serratia rubidaea, Enterococcus faecalis and almonella species*	*Salmonella*		
		3	22.17	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella species*	*Serratia rubidaea Salmonella*		
PYRase	3-Fluoroaniline	1	4.31	*Serratia rubidaea,Enterobacter cloacae* and *Salmonella species*	*Serratia rubidaea Salmonella*	0.0049	0.0163
		2	5.42	*Serratia rubidaea, Enterococcus faecalis and Salmonella species*	*Salmonella s*		
		3	2.43	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella species*	*Serratia rubidaea Salmonella*		

Table 7.13 liberated VOCs and isolated pathogens in Claxton blue cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	3.022	*Serratia rubidaea, Enterococcus faecalis* and *Enterobacter cloacae*	*Serratia rubidaea*	0.0140	0.0467
		2	1.97	Serratia rubidae and Enterococcus faecalis	Serratia rubidaea		
		3	2.92	Serratia rubidaea and Enterococcus faecalis	Serratia rubidaea		
α- Galactosidase	Phenol	1	27.82	*Serratia rubidaea, Enterococcus faecalis* and *Enterobacter cloacae*	*Serratia rubidaea*	0.0451	0.1503
		2	26.56	Serratia rubidaea and Enterococcus faecalis	Serratia rubidaea		
		3	28.11	Serratia rubidaea and Enterococcus faecalis	Serratia rubidaea		
PYRase	3-Fluoroaniline	1	2.94	*Serratia rubidaea, Enterococcus faecalis* and *Enterobacter cloacae*	*Serratia rubidaea*	0.0049	0.0163
		2	2.22	Serratia rubidaea and Enterococcus faecalis	Serratia rubidaea		
		3	2.35	Serratia rubidaea and Enterococcus faecalis	Serratia rubidaea		

Table 7.14 liberated VOCs and isolated pathogens in spiked Claxton blue cheese incubated with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. stanley* 10^4 CFU/mL (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 Esterase	2-Chlorophenol	1	3.58	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella*	*Serratia rubidaea* and *Salmonella*	0.0140	0.0467
		2	1.82	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella*	*Serratia rubidaea Salmonella*		
		3	3.53	*Serratia rubidaea, Enterococcus faecalis and Salmonella*	*Serratia rubidaea* and *Salmonella*		
α- Galactosidase	Phenol	1	26.00	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella*	*Serratia rubidaea* and *Salmonella*	0.0451	0.1503
		2	25.43	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella*	*Serratia rubidaea Salmonella*		
		3	24.85	*Serratia rubidaea, Enterococcus faecalis and Salmonella*	*Serratia rubidaea* and *Salmonella*		
PYRase	3-Fluoroaniline	1	3.14	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella*	*Serratia rubidaea* and *Salmonella*	0.0049	0.0163
		2	2.77	*Serratia rubidaea, Enterococcus faecalis* and *Salmonella*	*Serratia rubidaea Salmonella*		
		3	2.71	*Serratia rubidaea, Enterococcus faecalis and Salmonella*	*Serratia rubidaea* and *Salmonella*		

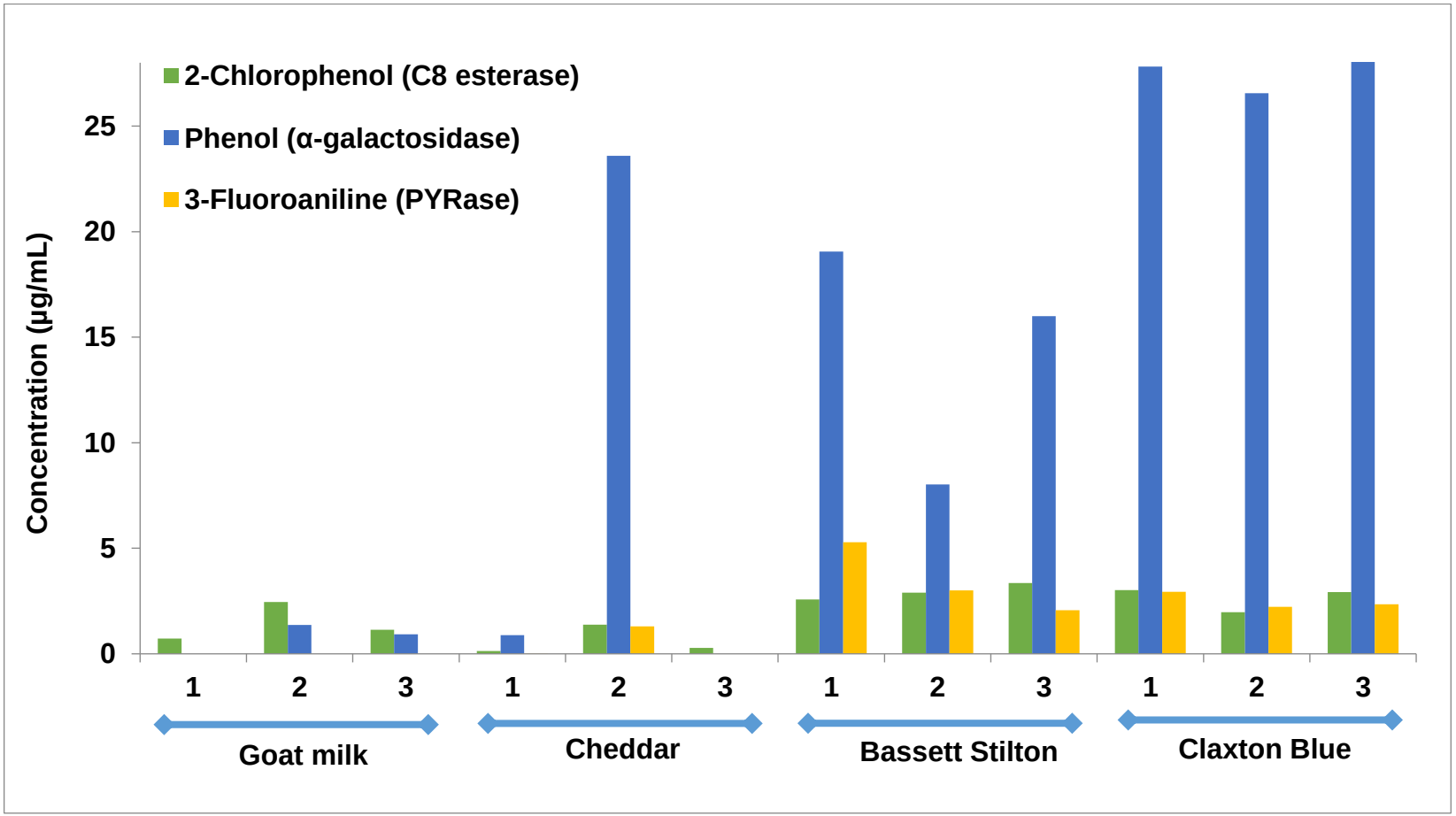


Figure 7.3 VOCs profiles for individual un-spiked cheese samples with antibiotics by HS-SPME GC/MS

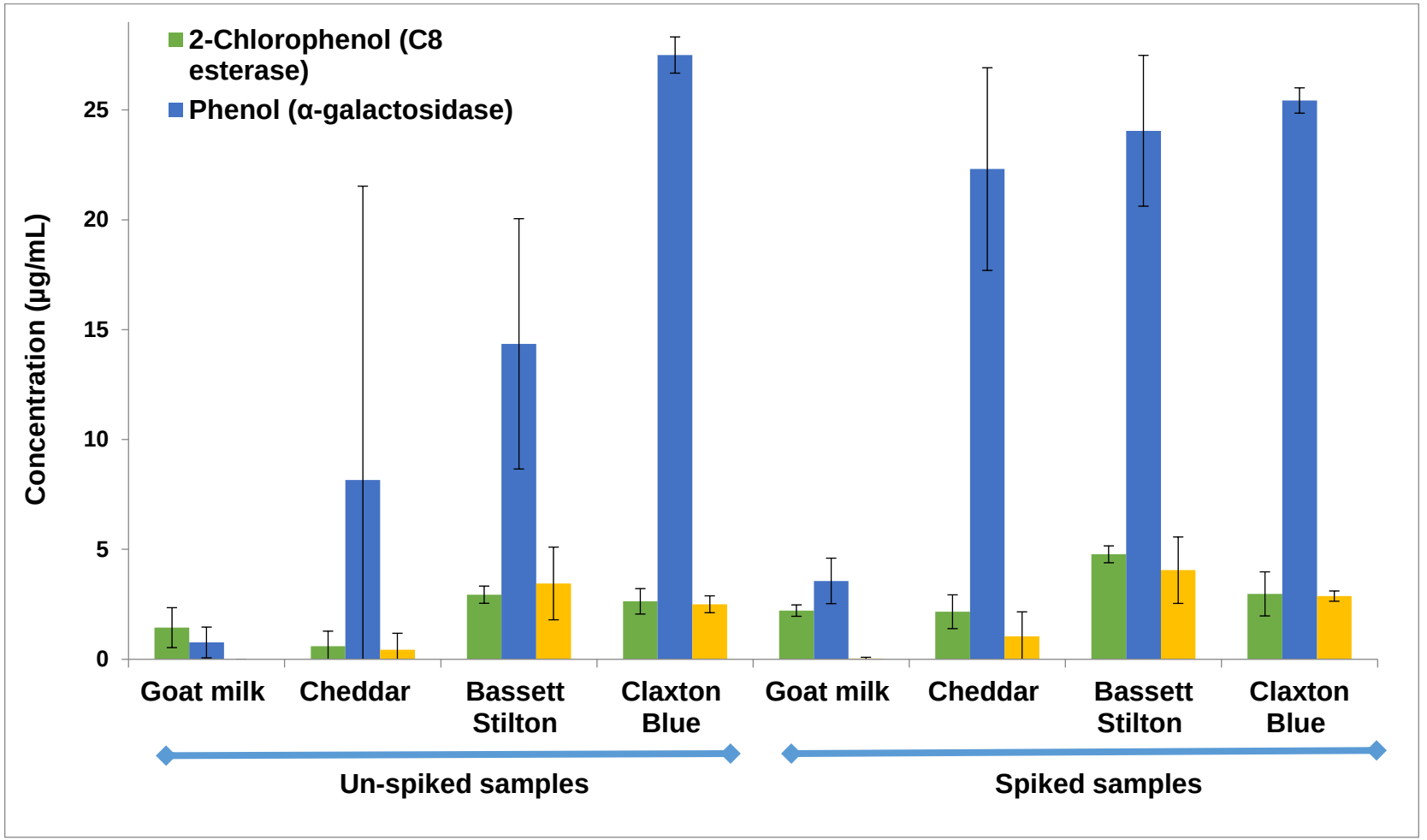


Figure 7.4 VOCs profiles for cheese samples with antibiotics by HS-SPME GC/MS

The C-8 esterase activity (2-chlorophenol) could be due to the presence of *Enterococcus faecalis, Serratia rubidaea* and *Klebsiella oxytoca* (Section 7.6). The PYRase activity (3-fluoroanaline) is due to the presence of *Serratia rubidaea* and *Klebsiella oxytoca,* while the α-galactosidase activity and the liberated phenol are evidently due to *Klebsiella oxytoca* (Section 7.6). In Claxton Blue cheese samples, the isolated resistant bacteria were *Serratia rubidaea, Enterococcus faecalis* and *Enterobacter cloacae*. These bacteria reacted with the enzyme substrates and liberated the three VOCs those detected in the samples.

7.5 Vancomycin and novobiocin resistant milk and cheese isolates

Adding vancomycin (5 mg/L) and novobiocin (10 mg/L) to samples of pasteurized milk and cheese made from pasteurized milk did not increase the specificity of the method as expected. Nonetheless, adding these antibiotics has made some differences in the isolated bacteria from milk and cheese samples as can be seen in Table 7.15. The Gram negative bacteria *Acinetobacter spp., Escherichia coli, and Proteus vulgaris* were not detected in milk and cheese samples when vancomycin (5 mg/L) and novobiocin (10 mg/L) were used. These bacteria seem to be inhibited by the antibiotics used or originally not present in the samples.

As the studied milk and cheese samples with and without antibiotics are the same type but they were collected on different days. *Streptococcus salivarius* and *Enterococcus faecalis* were isolated with and without use of the antibiotics. While, *Serratia rubidaea* and *Enterococcus faecium* were detected in cheese samples with the use of the antibiotics. These isolates shown to be vancomycin (5 mg/L) and novobiocin (10 mg/L) resistant.

Table 7.15 Resistant bacteria isolated in milk and cheese samples incubated with vancomycin and nivobiocin and identified using MALDI-TOF

Food type	Isolated on CLED agar plates with antibiotics	Isolated on CLED agar plates without antibiotics
Whole milk	*Streptococcus salivarius* *Enterobacter cloacae*	*Streptococcus salivarius*
Semi skimmed milk	*Enterobacter cloacae*	*Streptococcus salivarius*
Full cream milk	*Enterobacter cloacae,* *Enterococcus faecalis* *Streptococcus salivarius*	*Acinetobacter sp. and* *Enterococcus faecalis*
Goat milk cheese	*Enterooccus faecium*	*Enterococcus faecalis*
Cheddar Cheese	*Enterococcus faecalis* *Enterococcus species* *Cronobacter sakazakii*	*Escherichia coli,* *Proteus vulgaris*
Bassett Stilton cheese	*Serratia rubidaea Enterococcus faecalis Enterobacter cloacae,* *Klebsiella oxytoca* *Providencia rettgeri*	*Acinetobacter sp.,* *Enterococcus sp.* *Enterococcus faecalis*
Claxton Blue cheese	*Serratia rubidaea* *Enterococcus faecalis* *Enterobacter cloacae*	*Acinetobacter sp*

Many *Enterococci* species have naturally occurring resistances, while some are acquired resistances (Murray, 1990). The presence of antibiotic resistant *Enterococcus spp*. is due to the increase in the use of antibiotics both in human health care system and in agriculture as animal growth promoters (Aarestrup, 2000; Mannu *et al*., 2003). Consequently, the presence of the resistant enterococcal flora in some raw food types are dominated by *E. faecalis*. and *E. faecium*, (Klein *et al*., 1998). Antibiotic resistant *Enterococci* were present in different food items, including raw milk cheese (Emmenthal, Appenzell, Gruyere, Tilsit and soft cheeses) (Baumargartner *et al*., 2001). A study (Chajęcka-Wierzchowska *et al*., 2012) was reported that isolated of *Enterococcus faecalis* (44 strains), *Enterococcus faecium* (32 strains) or *Enterococcus spp.* (16 strains) out of 92 *Enterococcus* strains from foods of animal origin (cheese and meat). This study investigated the susceptibility of these enterococcal strains to

15 selected antibiotics commonly used in human therapies including vancomycin. The susceptibility tests were determined using the disk diffusion method (vancomycin 30 µg) all the investigated strains were sensitive to vancomycin. Another study (Giraffa and Sisto, 1997) of twenty strains of enterococci, either *Enterococcus faecium* or *E. faecalis*, isolated from different cheeses investigated the resistance of these strains to vancomycin in liquid medium and showed MIC values ranging from less than 1 to 4 µg / mL. It was concluded that the vancomycin resistant enterococcal species are rarely found in dairy products. In addition, a study (Franz *et al*., 2001) of the antibiotic susceptibility of enterococci isolated mostly from cheeses (48 *Enterococcus faecium* and 47 *Enterococcus faecalis*) found that, all *E. faecalis* strains and all but one *E. faecium* strain were susceptible to vancomycin. What is surprising is that, in the studied milk and cheese samples the *E. faecalis* that expected to inhibit by using a recommended MIC of 5 mg/L vancomycin (Figure 7.5) are still able to grow and isolate. However, this finding is in line with those of previous studies where enterococci are well documented in dairy foods (Bhardwaj *et al*., 2008) were *E. faecalis* species had been accounted a vancomycin resistance gene which was reported to be resistant to low levels of vancomycin (16 µg/mL) (Murray, 1997; Moellering, 1998; Cetinkaya *et al*., 2000). Also *E. faecalis* was reported to be resistant to novobiocin (12.5 µg/mL) (Patiño *et al*., 2005).

The effect of vancomycin on expression of *Streptococcus spp* is strain dependent as some *Streptococcus spp.* are reported to be susceptible to vancomycin (Barry *et al*., 1986); thus, the isolated *Streptococcus salivarius* are counted as vancomycin and novobiocin resistant species.

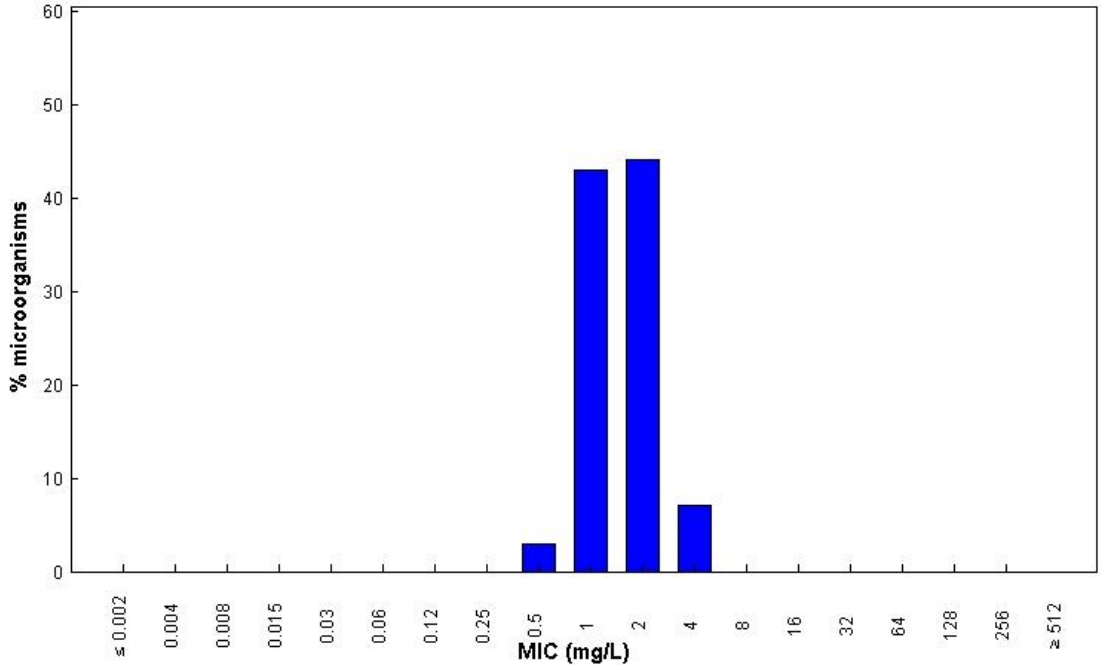


Figure 7.5 The MIC of vancomycin / *Enterococcus faecalis* (https://mic.eucast.org/Eucast2/regShow.jsp?Id=1211, no date)

Cronobacter sakazakii, Enterobacter cloacae and *Klebsiella oxytoca* are pathogens known to be resistant to some antibiotics (Paterson, 2006; Kim and Wei, 2007) and their detection in milk and cheese samples indicated that they are emerging resistance to the vancomycin (5 mg/L) and novobiocin (10 mg/L). *Serratia and Providencia* are pathogens resistant to most antibiotics usually isolated from food and soil (Santos *et al*., 2015).

Some resistant isolated bacteria exhibited positive enzyme activity (C-8 esterase, α-galactosidase and PYRase), but could not be proved by the literature. For example, the detection of phenol (α-galactosidase) in goat milk cheese samples number 2 and 3 (Table 7.7) could not be linked to the isolated *Enterococcus faecium.* In addition, the enzymatic activities of some isolated bacteria shown unexpected results as have been previously reported. For example, the C-8 esterase activity of *Enterobacter cloacae* was reported to be

negative (Cooke *et al*., 1999). However, these bacteria were the only isolated species from semi-skimmed milk samples (Table 7.3) and whole milk samples 2 and 3 (Table 7.1), and 2-chlorophenol were detected in these samples as C-8 esterase activity. Therefore, investigation of enzyme activity on these isolated was needed. The next Section is a discussion of the results obtained as experimental test to the enzymatic activities (especially C-8 esterase) of representative isolated species.

7.6 Enzymatic study of representative of food isolated antibiotic-resistant bacteria

The experiment was carried out to demonstrate the C-8 esterase activity detected on some bacteria isolated from milk and cheese samples and to prove/disprove that the VOCs detected (particularly 2-chlorophenol) were liberated during bacterial enzymatic activities in the samples. Seven species representative of the antibiotic-resistant bacteria isolated from milk and cheese samples were tested, these include *Enterobacter cloacae* (NCTC 11936), *Enterococcus faecalis* (NCTC 775), *Enterococcus faecium* (NCTC 7171), *Streptococcus salivarius* (NCTC 8618), *Cronobacter sakazakii* (ATCC 29544), *Klebsiella oxytoca* (Wild strain), *Serratia marcescens* (NCTC 10211) (very closely related to *Serratia rubidaea*).

7.6.1 VOCs analysis results

The bacterial growth and sample preparation was as described in Section 3.6. The sampling and the VOCs analysis was as described in Section 3.7 and 3.9, respectively. The results of VOCs analysis are presented in Table 7.16 and Figure 7.6; Figure 7.7, Figure 8 and Figure 7.9. It is apparent from Table 7. 16 that all strains tested are C-8 esterase positive as 2-chlorophenol was detected using HS-SPME GC-MS. Gordon *et al*. (1988) reported negative C-8 esterase

activity to *Enterococcus faecium* however, this activity was detected (2-chlorophenol, 0.38 µg/mL ± 0.02) on *Enterococcus faecium* (NCTC 7171) even with poor growth level in RVS after overnight incubation at 37 ºC where the plate count test result in 12 colonies /mL. Due to the level of growth of *Enterococcus faecium* (NCTC 7171) in RVS the positive PYRase activity (Gordon *et al*., 1988) could not be detected, however the strain was tested in TSB and shows liberation of 18.6 ± 0.73 µg / mL of 3-fluoroanaline.

The α-galactosidase activity of *Enterococcus faecium* (NCTC 7171) was not detected in both media (RVS and TSB). Therefore, this result does not clarify the occurrence of α-galactosidase activity in goat milk cheese samples 2 and 3 (Table 7.7) as the *Enterococcus faecium* was the only strain isolated from these samples. Gordon *et al*., (1988) reported the positive PYRase activity to *Enterococcus faecalis,* however, with a good growth in the RVS (colonies ≥ 400), the representative strain *Enterococcus faecalis* (NCTC 775) shows no PYRase activity. This test experiment was repeated in TSB as growth media and the PYRase activity was detected (3-fluoroaniline was 34.5 ± 0.4 µg/mL). The absence of the PYRase activity in RVS of *Enterococcus faecalis* (NCTC 775) could be due to the effect of the ingredient of the RVS that could act as inhibitors on the enzyme production. No α- galactosidase activities were detected for *Enterococcus faecalis* (NCTC 775) in both RVS and TSB. In contrast, positive C-8 esterase activity *Enterococcus faecalis* (NCTC 775) were observed in RVS and TSB as seen in Table 7.14. The detected α- galactosidase and C-8 esterase of *Enterococcus faecalis* and *Enterococcus faecium* were not supported in literature.

No VOCs were detected of *Streptococcus salivarius* (NCTC 8618) samples in RVS. Since the RVS broth did not turn cloudy after overnight

incubation at 37 ºC and by the plating count test (no colonies observed in the plates incubation at 37 º C after 24 and 48 hours), it was concluded that, RVS inhibited the growth of *Streptococcus salivarius* (NCTC 8618). To study the enzymatic activities of this strain, it needs to grow in a general nutrient broth, such as, TSB. So the enzymatic activity of *Streptococcus salivarius* (NCTC 8618) was investigated in TSB after overnight incubation at 37 ºC. The observed result indicated that this strain is negative for PYRase and α- galactosidase while positive for C-8 esterase (5.5 ± 0.3 µg/mL). This result supports the detected results in whole milk sample number 1 (Table 7.1). However, even though, *Streptococcus salivarius* should be inhibited by RVS the isolation of *Streptococcus salivarius* in milk samples prepared in RVS could be due to excessively high inoculum as even one colony in 25 g of food sample would have resulted in a positive test (Klein *et al*., 1998).

Cronobacter sakazakii (ATCC 29544) had shown the highest C-8 esterase activities (6.96 ± 0.31 µg / mL) obtained in RVS. *Cronobacter sakazakii* (ATCC 29544) was also positive for PYRase and α- galactosidase (Table 7.14, and Figures 7.7, 7.8, 7.9). These positive activities are in accord with previous study (Muytjens and Van Druten, 1984). In RVS the PYRase activity of *Enterobacter cloacae* (NCTC 11936) was detected, but below the quantification limit (0.0163 µg/mL) and the α-galactosidase activity was quantifiable (9.21 µg/mL) with a standard deviation of ± 4.35 µg/mL. The second highest C-8 esterase activity (2.6 ± 0.42 µg/mL) in RVS was due to *Enterobacter cloacae* (NCTC 11936). Even though, this result is in contrast with the result reported by Cooke *et al*. (1999), it is supporting the findings in semi-skimmed milk samples (Table 7.3). The *Serratia marcescens* (NCTC 10211), is closely related to *Serratia rubidaea* that was isolated from Bassett Stilton cheese and Claxstone Blue cheese samples.

Table 7.16 VOCs profiles of representative species of antibiotic-resistant bacteria isolated from milk and cheese samples detected in RVS and TSB using HS-SPME-GC-MS (polar SPME fiber and polar GC column) (n = 3)

Bacteria	Growth in RVS	C-8 esterase 2-chlorophenol (µg/mL) Mean ± SD		α- Galactosidase phenol (µg/mL) Mean ± SD		PYRase 3-fluoroaniline (µg/mL) Mean ± SD	
		TSB	RVS	TSB	RVS	TSB	RVS
***Enterobacter cloacae* (NCTC 11936)**	Poor	10.2 ± 3.2	2.6 ± 0.4	27 ± 7	9.2 ± 4.3	0.12 ± 0.02	NQ
***Enterococcus faecalis* (NCTC 775)**	Good	15.7 ± 1.1	0.6 ± 0.1	ND	ND	34.5 ± 0.4	ND
***Enterococcus faecium* (NCTC 7171)**	Poor	7.4 ± 2.4	0.4 ± 0.02	ND	ND	18.6 ± 0.73	ND
***Streptococcus salivarius* (NCTC 8618)**	NG	5.5 ± 0.3	NG	ND	NG	ND	NG
***Cronobacter sakazakii* (ATCC 29544)**	Good	11.1 ± 1.8	7.0 ± 0.3	28.2 ± 2.2	29.3 ± 1.3	0.03 ± 0.01	0.1 ± 0.03
***Klebsiella oxytoca* (Wild strain)**	Good	1.7 ± 0.3	0.1 ± 0.02	18 ± 5	23.3 ± 1.4	ND	ND
***Serratia marcescens* (NCTC 10211) (*Serratia rubidaea*)**	Good	25.4 ± 2.1	0.6 ± 0.2	ND	ND	12.7 ± 0.3	0.5 ± 0.2

NG = no groth, ND = not detcted, NQ = not quantifiable, SD standard deviation

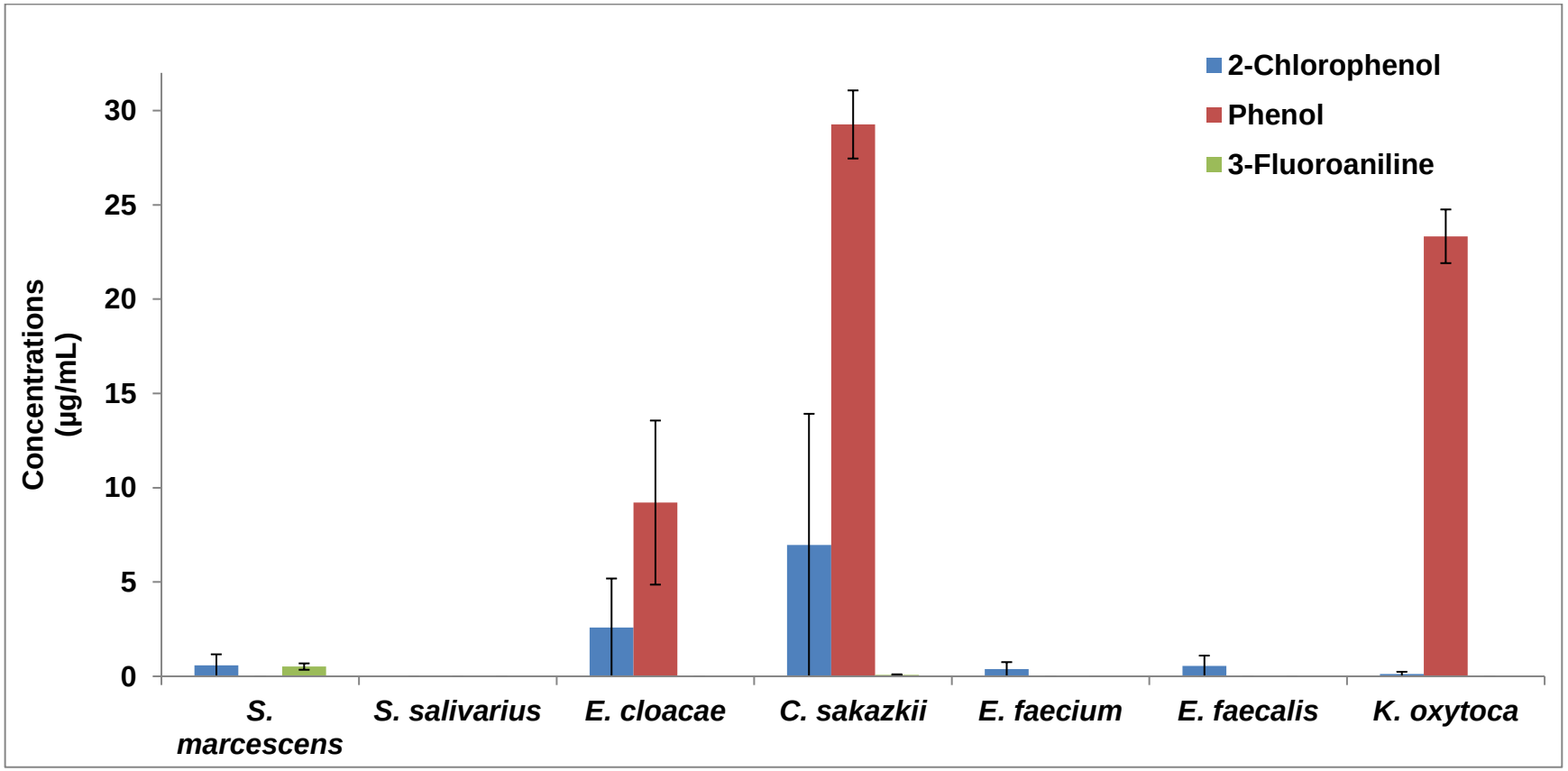


Figure 7.6 VOCs profiles of representative of food antibiotic-resistant bacteria in RVS

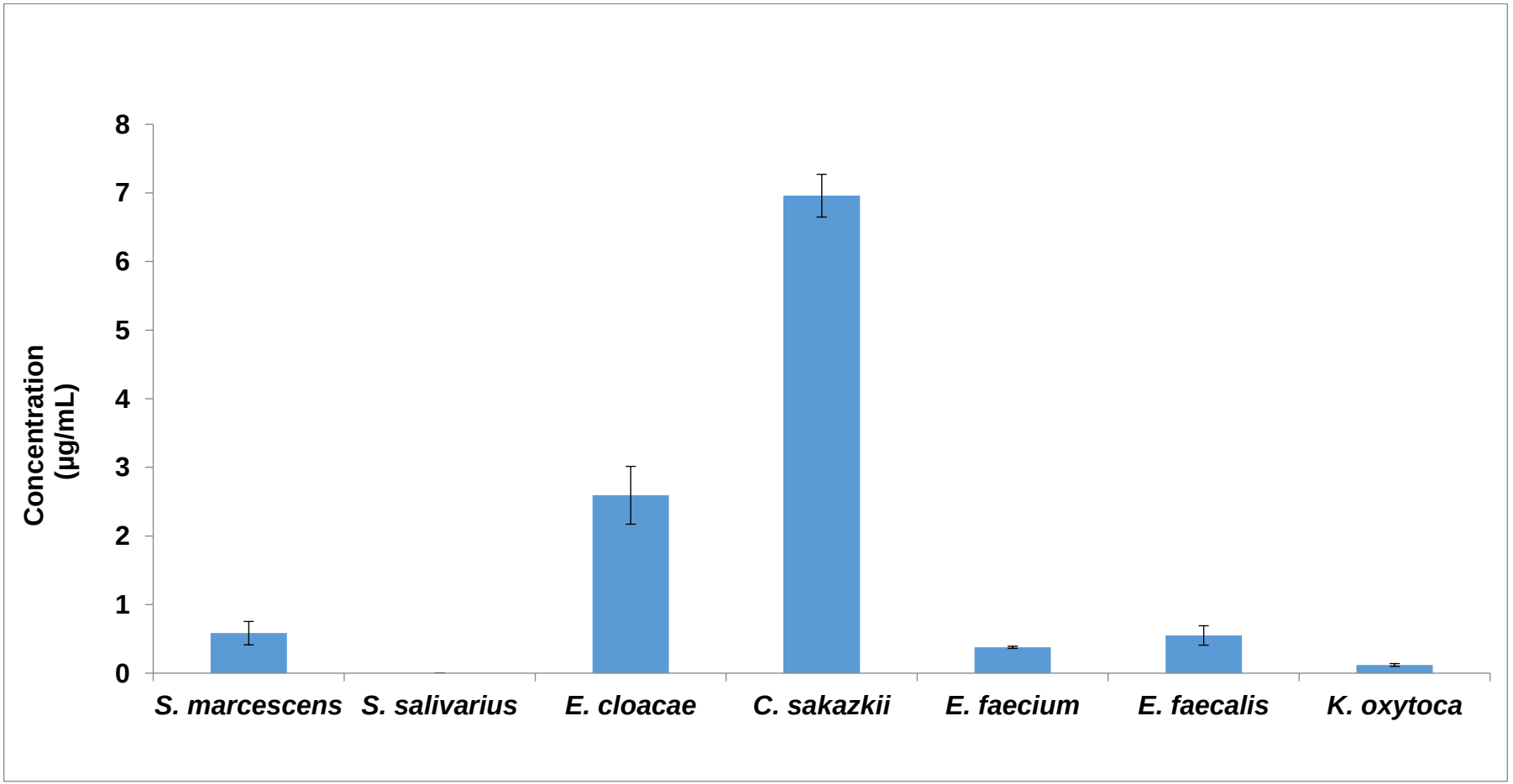


Figure 7.7 Concentration of 2-chlorophenol liberated during C-8 esterase activity in RVS

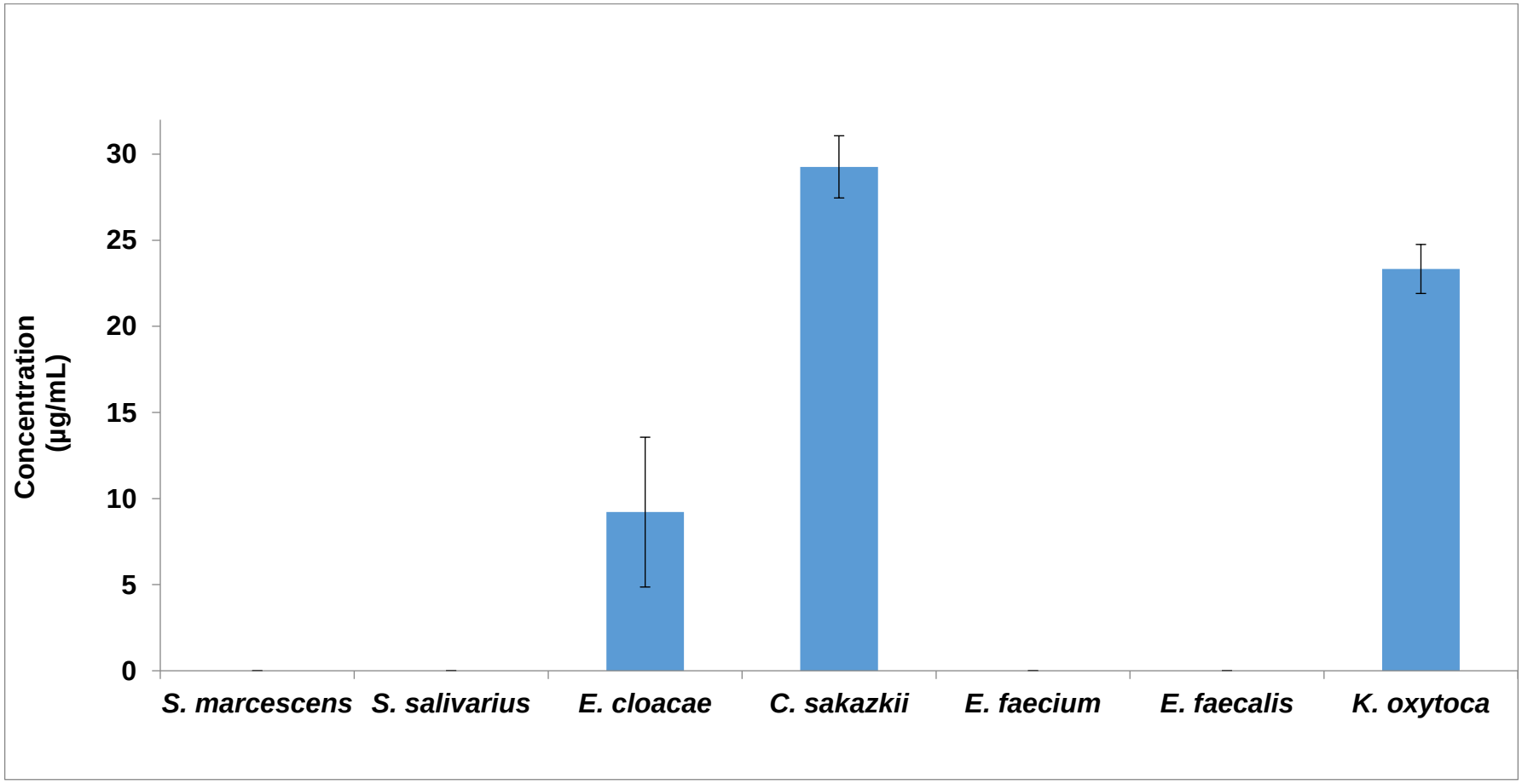


Figure 7.8 Concentration of phenol liberated during bacteria α-galactosidase activity in RVS

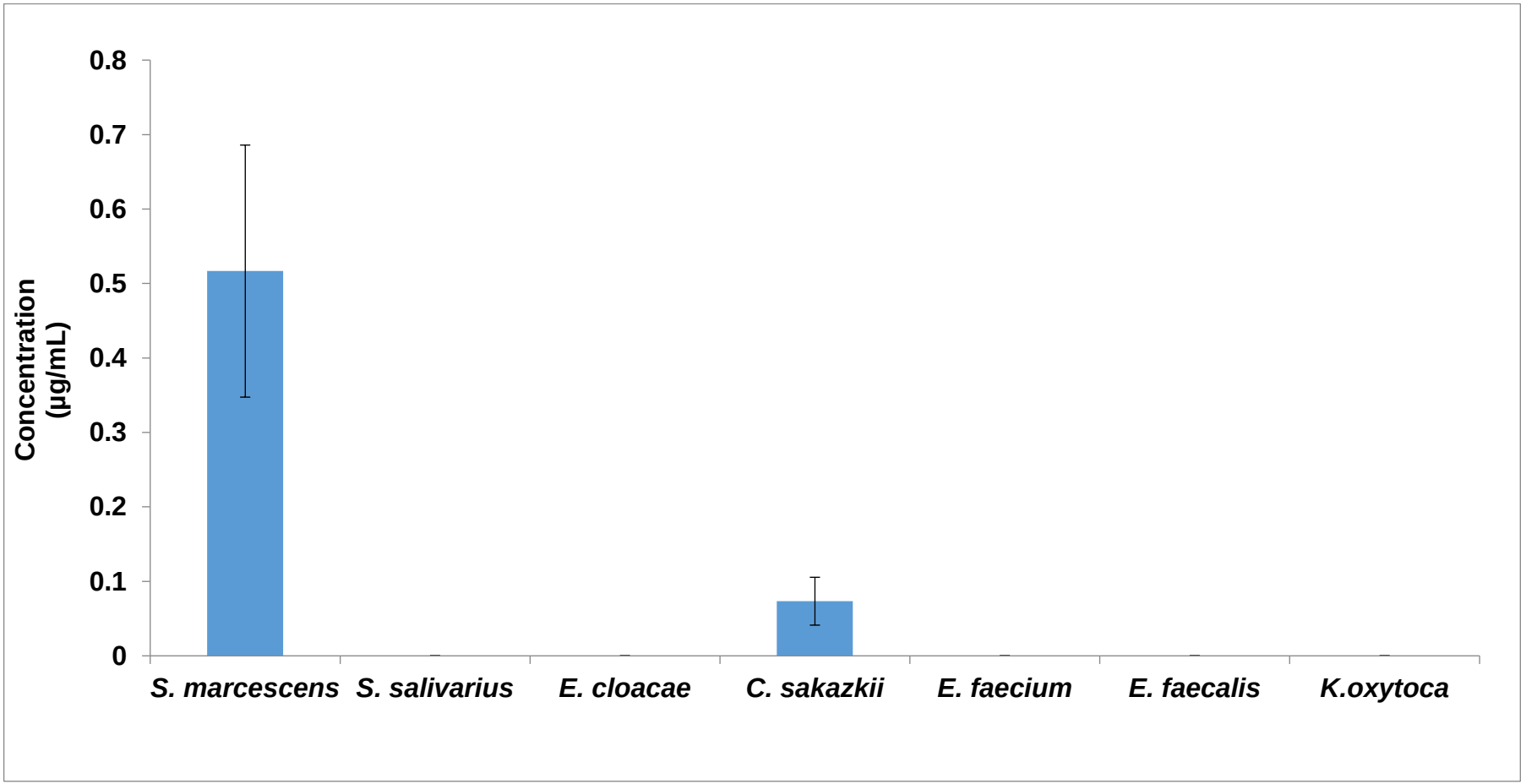


Figure 7.9 Concentration of 3-fluoroaniline liberated during bacteria PYRase activity in RVS

The C-8 esterase activity of *Serratia marcescens* (NCTC 10211) in RVS was detected and the detected 2-chlorophenol was 0.6 ± 0.2 µg/mL. However, this activity was reported to be negative (Freydiere and Gille, 1991; Cooke *et al*., 1999). The PYRase activity detected and the amount of 3-fluoroaniline was 0.5 ± 0.2 µg/mL. The detected positive PYRase activity was previously supported (Inoue *et al*., 1996). No α- galactosidase activity was detected for this strain as was previously reported (Freydiere and Gille, 1991).

The smallest C-8 esterase activity in RVS was observed for *Klebsiella oxytoca* (Wild strain) where the detected 2-chlorophenol was 0.12 ± 0.02 µg/mL. This strain showed the highest α- galactosidase activity among the tested species where the phenol concentration was 23.3 ± 1.4 µg/mL. No PYRase activity was detected for *Klebsiella oxytoca* (Wild strain). No information about the enzymatic activities of *Klebsiella oxytoca* (Wild strain) was obtained in the literature.

The detected C-8 esterase activity of some representative strains does not explain the occurrence of this activity in the milk and cheese samples studied. Future investigations on C-8 esterase activity of the representative bacteria therefore, are recommended. Also, to investigate whether the detected 2-chlorophenol was liberated by the isolated bacteria (specially *Enterobacter cloacae* and *Serratia marcescens*) and not due to the hydrolysis of 2-chlorophenyl octanoate during bacterial growth on RVS broth. Results of this investigation are discussed below.

7.6.2 Fluorescent study

The enzymatic study of representative food isolated antibiotic-resistant bacteria was carried out using a fluorogenic substrate 4-methylumbelliferyl caprylate. The sample preparation and the experimental details are as described

in Section 3.11.4.2. Excitation and emission characteristics of the fluorophore of 4-methylumbelliferyl caprylate substrate were scanned and installed in the instrument (350 and 575 nm, respectively). For quantitative analysis a calibration curve was obtained using 5 standards of 4-methylumbelliferone in ethanol ranging from 1-25 µg/mL and prepared in TSB solution. The calibration graph and the equation used for data calculation are as shown in Figure 7.10.

After overnight incubation at 37 ºC, the 4-methylumbelliferone was detected in all bacteria samples indicating the positive C-8 esterase activity in these strains (Table 7.17 and Figure 7.11). Therefore, even though Cooke *et al*. (1999) reported the negative C-8 esterase activity of *Enterobacter cloacae*, the findings in this study support the results obtained when the C-8 esterase activity was tested using 2-chlorophenyl octanoate. In addition, both results of C-8 esterase tests are supporting the findings in semi-skimmed milk samples (Table 7.3). Similarly, the C-8 esterase activity of *Serratia marcescens* was reported to be negative (Freydiere and Gille, 1991; Cooke *et al*., 1999). However, the representative strain *Serratia marcescens* (NCTC 10211) as tested in RVS and TSB with 2-chlorophenyl octanoate and the result showed positive C-8 esterase activity. In TSB, this strain was tested with 4-methylumbelliferyl caprylate and was observed to be C-8 esterase positive. The obtained results are consistent with data obtained in Bassett Stilton cheese and Claxstone Blue cheese samples.

Serratia marcescens (NCTC 10211), is closely related to *Serratia rubidaea* that was isolated from Bassett Stilton cheese and Claxstone Blue cheese samples. The C-8 esterase activity of *Serratia marcescens* (NCTC 10211) in RVS was detected and the detected 2-chlorophenol was 0.6 ± 0.2 µg/mL however, this activity was reported to be negative (Freydiere and Gille, 1991; Cooke *et al*., 1999).

In TSB the *Klebsiella oxytoca* (Wild strain) showed the highest level of C-8 esterase activity detected (Table 7.17), where with 2-chlorophenyl octanoate in RVS was detected as the lowest (Table 7.16). It is possible that the difference between the level of growth in TSB and RVS made the difference in the detected level of the C-8 enzyme activity.

Table 7.17 Concentrations of 4-methylumbelliferone ((µg/mL) liberated by bacteria during esterase activity in TSB at pH 7.6 detected using spectro-fluorometer (n = 3)

Bacteria	Concentrations (µg/mL) Mean ± SD (… ;…;…)
Serratia marcescens (NCTC 10211)	0.91 ± 0.59 (0.257; 1.08; 1.40)
Streptococcus salivarius (NCTC 8618)	42 ± 0.13 (1.34; 1.57; 1.34) 1.
Enterobacter cloacae (NCTC 11936)	1.84 ± 0.76 (1.16; 2.65; 1.71)
Cronobacter sakazakii (ATCC 29544)	1.84 ± 0.46 (1.42; 2.33; 1.78)
Enterococcus faecium (NCTC 7171)	2.05 ± 0.22 (1.80; 2.14; 2.21)
Enterococcus faecalis (NCTC 775)	2.12 ± 0.14 (2.13; 2.26; 1.97)
Klebsiella oxytoca (Wild strain)	2.50 ± 0.60 (2.87; 2.83; 1.81)

SD = standard diviation; (… ;…;…) = the three individual concentration

7.6.3 Study of the variation on hydrolysis of esterase substrates

This experiment was made to investigate the hydrolysis of the enzyme substrate 2-chlorophenyl octanoate and 4-methylumbelliferyl caprylate on aqueous phase during bacterial growth on RVS broth. Eight blank samples of 2-chlorophenyl octanoate (100µg/L) were prepared on 10 mL RVS broth. The samples were incubated for 18-24 hours at 37 °C and subjected to volatile profiling via HS-SPME-GC-MS.

The concentration of 2-chlorophenol was liberated through hydrolysis process and/or found as an impurity of the substrate was determined. The hydrolysis of 2-chlorophenyl octanoate 100 µg / mL resulted in detection of 0.28 ± 0.02 µg / mL 2-chlorophenol. This concentration was considered small for the detected concentration liberated by bacteria, which ranged from 0.1-10 µg / mL.

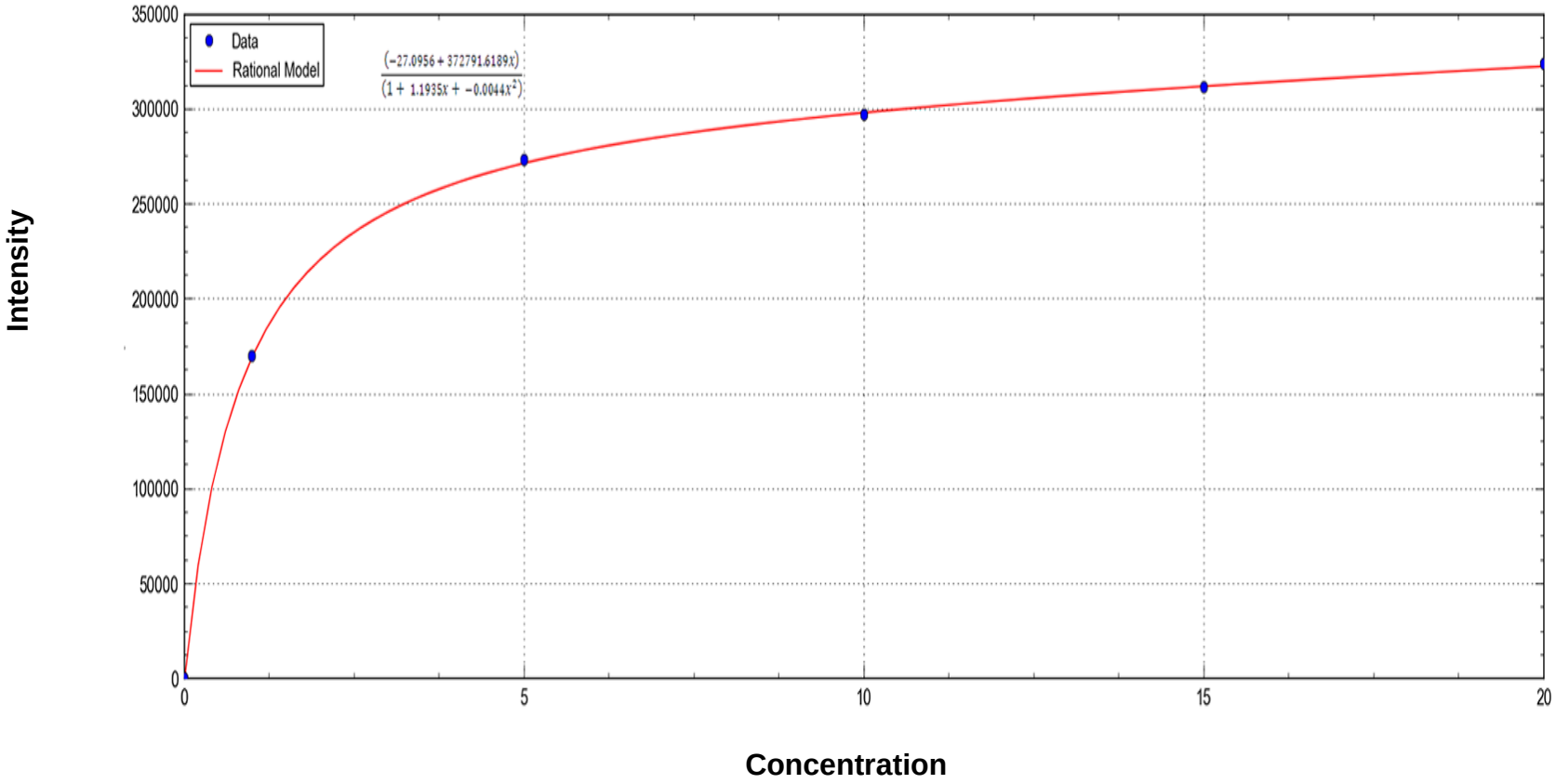


Figure 7.10 Calibration curve of 4-methylumbelliferone

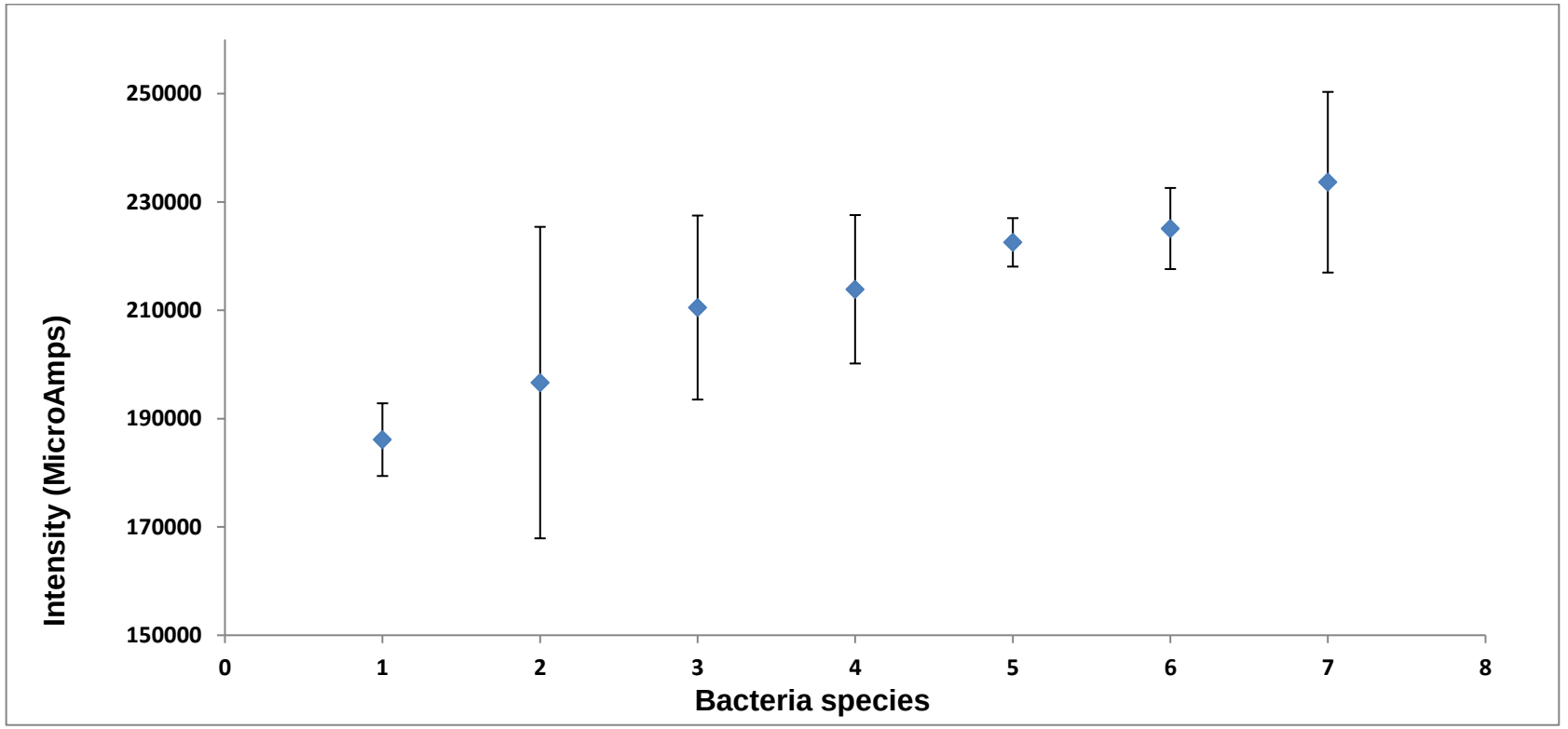


Figure 7.11 The C 8 esterase activity detected using 4-methylumbelliferyl caprylate

1 = *Serratia marcescens* (NCTC 10211), 2 = *Streptococcus salivarius* (NCTC 8618), 3 = *Enterobacter cloacae* (NCTC 11936), 4 = *Cronobacter sakazakii* (ATCC 29544), 5 = *Enterococcus faecium* (NCTC 7171), 6 = *Enterococcus faecalis* (NCTC 775), and 7 = *Klebsiella oxytoca* (Wild strain)

As in an earlier study, Bobey and Ederer (1981) found that the 4-methylumbelliferyl substrates hydrolyzed spontaneously in the pH range 6.0 to 7.0, resulting in nonspecific fluorescence. This experiment was investigated to avoid any false positive results that could be detected when investigating the bacteria esterase activities with the substrate 4-methylumbelliferyl caprylate. This substrate was prepared in ethanol at a concentration of 100,000 µg / mL. Of this solution 5 samples 100 µg/mL were prepared in 10 mL TSB after adjusting the pH of the broth to the range of 6-7 with 1M HCl. As this substrate precipitated (cloudy) in the broth, 0.075 g of Tween 20 was added to the TSB before adding the substrate. After overnight incubation at 37 °C, the fluorescence emission was measured at wavelength of 575 nm. The hydrolysis of 4-methylumbelliferyl caprylate substrate 100 µg / mL at pH 7.3-7.7 resulted in 0.099 ± 0.020 µg / mL of 4-methylumbelliferone and 0.078 ± 0.014 µg / mL at pH 6.02-7.02. There was no significant difference between the hydrolysis of the substrate at both pH values, as the calculated p-value was 2.78, which is greater than 0.05 at 95% confidence level.

The conclusion made of these experiments is that, the detected 2-chlorophenol in bacteria samples grown in RVS is due to reaction of the bacterial enzymes with the substrate 2-chlorophenyl octanoate and similarly the detected 4-methylumbelliferone is due to the bacteria enzymatic activity and not to hydrolysis of the substrates.

7.7 Summary and future work

It is obvious from all these experiments' results, that the specificity of the developed *Salmonella* detection method did not solve as required yet. Adding antibiotics such as vancomycin (5 mg/L) and novobiocin (10 mg/L) to the samples of pasteurized milk and cheese made from pasteurized milk during the period of

incubation in *Salmonella* selective broth (RVS) are rather disappointing. Because vancomycin (5 mg/L) and novobiocin (10 mg/L) did not inhibit bacteria however, has made some differences in the isolated bacteria (Table 7.13).

The most obvious finding to emerge from study of representative of food isolated antibiotic-resistant bacteria is that, all the studied strains shown C-8 esterase activity. These findings will be crucial in elucidating the 2-chlorophenol detected in milk and cheese samples. Whole milk samples and semi-skimmed milk samples showed less variation in the isolated bacteria and simpler interpretation results than full cream milk and cheese samples.

As most isolated species are either very resistant to many agents or can develop resistance in presence of antibiotics, the choice of appropriate antimicrobial agents and the effective concentration are complicated. In addition, the results of investigations done by Klein *et al*. (1998) on raw minced beef and pork found that *Enterococci* isolated from clinical samples exhibit different resistance patterns than *Enterococci i*solated from meat. Cetinkaya *et al*. (2000) supported this finding. Therefore, the MIC of food isolates is different from clinical isolates which make the solution is more complicated. However, the specificity of the developed assay for detection of *Salmonella* in food samples is still the main issue in the project and concerning to improve it is continuing.

It would be interesting to assess the effect of other concentration of vancomycin and novobiocin on the inhibition of pathogens specially *Enterococcus faecalis* and *Enterococcus spp*. To estimate the digestion of the food samples (enrichment step) in RVS broth as an alternative to BPW further investigation and experimentation are needed. Furthermore, some other parameters are need to be studied, such as; digestion and incubation time in

order to decrease the inoculum effect of other pathogens. More broadly testing some other bacterial inhibitors that would be save for *Salmonella* and could control or inhibit other bacteria growth (e. g lithium chloride). The next chapter is discussions to results obtained from these suggested investigations.

Chapter 8: Parameters evaluation in the detection method of *Salmonella*

8.1 Introduction

One unanticipated finding in the last chapter was the isolation of *Enterococcus faecalis, Enterooccus faecium*, *Enterococcus spp*. and *Staphylococcus epidermidis* on milk and cheese samples when incubated with vancomycin 5 mg/L and novobiocin 10 mg/L as these isolates are susceptible to these concentrations (Antimicrobial: Vancomycin, no date). The isolation of such bacteria could be due to the high-bacterial-density that is often difficult to eradicate and this inoculum appears to influence the activity of vancomycin and novobiocin (Laplante and Rybak, 2004). This chapter provides descriptions and discussions to the results of experiments investigating the effect of adding antibiotics to milk and cheese samples during the enrichment and incubation process in order to obtain better specificity. Evaluation of some more parameters that influence the *Salmonella* detection method is given below.

8.2 Evaluation of the digestion / enrichment process

A study of the digestion process of milk and cheese samples using the enrichment media BPW and RVS with and without addition of vancomycin (5 mg/L) and novobiocin (10 mg/L) was carried out. The sample preparation was as described in Section 3.11.2. The liberated VOCs extracted, separated and identified using HS-SPME GC/MS (Section 3.7) and the bacteria were isolated and identified as described in Section 3.8.2.

8.2.1 Pre-enrichment process in non-selective medium BPW

Whole milk samples and cheddar cheese samples were subjected to a comparison study. The milk and the cheese samples were pre-enriched (digested) in liquid enrichment media BPW at 37 ºC for 16-20 h without and with

addition of vancomycin (5 mg/L) and novobiocin (10 mg/L). The obtained results are discussed below.

8.2.1.1 Milk samples

Results of analyzing whole milk samples using BPW as a pre-enrichment medium without adding antibiotics are presented in Table 8.1. C-8 esterase activity was detected in milk samples along with isolation of *Streptococcus salivarius ssp. thermophilus*. *Streptococcus salivarius ssp. thermophilus* has an optimal growth temperature range of 35 - 42 °C and it has been found in fermented milk products (Kiliç *et al*., 1996). Presence of these species are likely to be related to the long digestion time (16 h) at 37 ºC. It can thus be suggested that reducing the enrichment time may result in no isolation of these species.

The results of spiked whole milk samples digested in BPW without addition of antibiotics are shown in Table 8.2. What is interesting about the data in this table is that *S. stanley* and *Streptococcus salivarius ssp. thermophilus* were isolated however, it is somewhat surprising that the amount of liberated phenol was noted in this condition to be smaller than what was expected and usually detected in the positive control samples. Therefore, one can conclude the presence of *Streptococcus salivarius ssp. thermophilus* affect in the detection of α- galactosidase of *S. stanley* somehow. As the isolation of pathogens from milk samples digested in BPW without adding antibiotics remain of concern as it effects the specificity of *Salmonella* detection method. It was therefore decided to add the antibiotics to the enrichment medium BPW and repeat the analysis at the same conditions in order to obtain better specificity. Addition of the antibiotics to whole milk samples during the digestion (enrichment step) did not inhibit the growth of pathogens present.

Table 8.1 liberated VOCs and isolated pathogens in whole milk samples digested in BPW and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	2.9	*Streptococcus salivarius ssp. thermophilus*	NG	0.0140	0.046
		2	2.7	*Streptococcus salivarius ssp. thermophilus*	NG		
		3	2.6	*Streptococcus salivarius ssp. thermophilus*	NG		
α- Galactosidase	Phenol	1	ND	*Streptococcus salivarius ssp. thermophilus*	NG	0.0451	0.1503
		2	ND	*Streptococcus salivarius ssp. thermophilus*	NG		
		3	ND	*Streptococcus salivarius ssp. thermophilus*	NG		
PYRase	3-Fluoroaniline	1	ND	*Streptococcus salivarius ssp. thermophilus*	NG	0.0049	0.0163
		2	ND	*Streptococcus salivarius ssp. thermophilus*	NG		
		3	ND	*Streptococcus salivarius ssp. thermophilus*	NG		

ND = not detected, NG = no growth

Table 8.2 liberated VOCs and isolated pathogens in spiked whole milk samples digested in BPW and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Spiked *S. Stanley* (1×10^4 CFU/mL) (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	4.7	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*	0.0140	0.046
		2	5.1	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*		
		3	5.0	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*		
α- Galactosidase	Phenol	1	0.74	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*	0.0451	0.1503
		2	0.85	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*		
		3	0.75	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*		
PYRase	3-Fluoroaniline	1	ND	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*	0.0049	0.0163
		2	ND	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*		
		3	ND	*Salmonella and Streptococcus salivarius ssp. thermophilus*	*Salmonella*		

ND = not detected, NG = no growth

As shown in Table 8.3 *Acinetobacter baumannii* were isolated on CLED agar plates on one of the whole milk samples with no VOCs detected in all the samples. *Acinetobacter baumannii* has been previously isolated from milk samples (Jayarao and Wang, 1999) and have emerged as an important pathogen, causing infections in severely ill patients (Villegas and Hartstein, 2003). *Acinetobacte*r species are known to be C-8 esterase positive (Freydiere and Gille, 1991), and PYRase positive (Bomicino *et al*. 2007) however, the VOCs were not detected. Not detecting the VOCs could be either due to the poor growth of the species on RVS and/or the liberated VOCs are of an undetectable level.

8.2.1.2 Cheese samples

As shown in Table 8.4 *Enterococcus species* and *Enterococcus faecalis* were isolated on cheddar cheese samples digested in non-selective pre-enrichment medium BPW without addition of vancomycin (5 mg/L) and novobiocin (10 mg/L). As a result of presence of these species C-8 esterase and PYRase activities were detected. Spiked cheddar cheese samples represent an expected result. The results in Table 8.5 were obtained after addition of vancomycin (5 mg/L) and novobiocin to cheddar cheese digested in BPW. No bacterial inhibition was detected in these samples as *Lactobacillus curvatus* were isolated and C-8 esterase were detected. Enzymatic activities of some isolated bacteria from food samples were summarised in Table 8.6.

Therefore, adding the antibiotics to the food samples in the enrichment step did not overcome the overgrowth of the pathogens. What stands out in milk and cheese results is the problem that often arises in pre-enriched samples which is the overgrowth of enteric flora on the enrichment growth media that result in the inoculum effect (IE).

Table 8.3 liberated VOCs and isolated pathogens in whole milk samples digested in BPW with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	ND	*NG*	NG	0.0140	0.046
		2	ND	*NG*	NG		
		3	ND	*Acinetobacter baumannii*	*NG*		
α- Galactosidase	Phenol	1	ND	*NG*	NG	0.0451	0.1503
		2	ND	*NG*	NG		
		3	ND	*Acinetobacter baumannii*	*NG*		
PYRase	3-Fluoroaniline	1	ND	*NG*	NG	0.0049	0.0163
		2	ND	*NG*	NG		
		3	ND	*Acinetobacter baumannii*	*NG*		

ND = not detected, NG = no growth

This IE could not be overcome by adding vancomycin 5 mg/L and novobiocin 10 mg/L in digestion and incubation steps. Concluded that non-selective pre-enrichment medium BPW is not supporting the specificity of *Salmonella* detection method and need to use another medium.

8.2.2 Selective enrichment process using RVS

The isolation and detection of *Salmonella* species from food samples was as recommended by the International Standards Organisation (ISO) 6579. In this method the use of BPW in the enrichment step was recommended followed by incubation of the enriched food sample in RVS broth for 18-24 h at 37 °C before the analysis. As mentioned above the struggle that often arises in pre-enriched samples using BPW is the overgrowth of target *Salmonellae* by competitive enteric flora on the enrichment growth media which result in the inoculum effect (IE) that could not be overcome by using vancomycin 5 mg/L and novobiocin 10 mg/L (Laplante and Rybak, 2004).

From our observations, this problem often arises in pre-enriched samples where, *Lactobacillus curvatus*, *Acinetobacter baumannii*, *Enterococcus species*, *Enterococcus faecalis*, *Cronobacter sakazakii* are dominant in milk and cheese samples. For that reason, it was absolutely necessary to deviate from the standard method. The *Salmonella* selective broth RVS is well known as highly effective for recovery of *Salmonella* from foods with a high level of background contamination (Public Health England, 2014). Therefore, RVS chosen to be use as enrichment medium to help in overcome of the IE in enriched milk and cheese samples.

Table 8.4 liberated VOCs and isolated pathogens in cheddar cheese digested in BPW and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	5.5	*Enterococcus species*	NG	0.0140	0.046
		2	7.12	*Enterococcus faecalis*	NG		
		3	5.68	*Enterococcus faecalis*	NG		
α- Galactosidase	Phenol	1	ND	*Enterococcus species*	NG	0.0451	0.1503
		2	ND	*Enterococcus faecalis*	NG		
		3	ND	*Enterococcus faecalis*	NG		
PYRase	3-Fluoroaniline	1	0.13	*Enterococcus species*	NG	0.0049	0.0163
		2	0.13	*Enterococcus faecalis*	NG		
		3	0.05	*Enterococcus faecalis*	NG		

ND = not detected, NG = no growth

Table 8.5 liberated VOCs and isolated pathogens in cheddar cheese digested in BPW with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on TSA	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	3.51	*Lactobacillus curvatus*	NG	0.0140	0.046
		2	4.97	*Lactobacillus curvatus*	NG		
		3	2.45	NG	*NG*		
α- Galactosidase	Phenol	1	ND	*Lactobacillus curvatus*	NG	0.0451	0.1503
		2	ND	*Lactobacillus curvatus*	NG		
		3	ND	NG	*NG*		
PYRase	3-Fluoroaniline	1	ND	*Lactobacillus curvatus*	NG	0.0049	0.0163
		2	ND	*Lactobacillus curvatus*	NG		
		3	ND	NG	*NG*		

ND = not detected, NG = no growth

Table 8.6 Enzymatic activities of some isolated bacteria from food samples (ATCC, NCTC, and wild are the tested strains)

Bacteria	Type	C-8 esterase	α- Galactosidase	PYRase
Acinetobacter sp.	Gm -	**Positive** (Freydiere and Gille, 1991)	**Negative** (API strips)	**Variable** (depending on species**)** (Bomicino *et al*. 2007)
Aeromonas species	Gm -	**Positive** (Awan *et al*., 2005)	**Negative** (Awan *et al*., 2005)	**Unknown**
Cronobacter sakazakii • (ATCC 29544)	Gm -	**Positive** (Muytjens and Van Druten, 1984), **Positive** (Own work in RVS and TSB)	**Positive** (Muytjens and Van Druten,1984), **Positive** (Own work in RVS and TSB)	**Positive** (Own work in RVS and TSB)
Cuprividus species	Gm -	**Unknown**	**Unknown**	**Unknown**
Enterobacter cloacae • (NCTC 11936)	Gm -	**negative** (Cooke *et al*., 1999), **Positive** (Own work in RVS and TSB)	**Positive** (Own work in RVS and TSB)	**Positive** (Own work, in RVS and TSB)
Enterococcus faecalis • (NCTC 775)	Gm +	**Positive** (Own work, in RVS and TSB)	**Negative** (Own work, in RVS and TSB)	**Positive** (Gordon *et al*., 1988), **Negative** (Own work, in RVS) **Positive** (Own work, in TSB)
Enterococcus faecium • (NCTC 7171)	Gm +	**Positive** (Own work, in RVS and TSB)	**Negative** (Own work, in RVS and TSB)	**Positive** (Gordon *et al*., 1988), **Positive** (Own work, in TSB) **Negative** (Own work, in RVS)
Enterococcus sp	Gm +	**Unknown**	**Unknown**	**positive** (Gordon *et al*.)
Escherichia coli	Gm -	**Negative** (Dealler *et al*.1992)	**Positive** (Kämpfer *et al*., 1991)	**Negative** (Freydiere and Gille, 1991)
Klebsiella oxytoca • (Wild strain)	Gm -	**Positive** (Own work, in RVS and TSB)	**Positive** (Own work, in RVS and TSB)	**Negative,** (Own work, in RVS and TSB)

Continue, Table 8.6 Enzymatic activities of some isolated bacteria from food samples

Bacteria	Type	C-8 esterase	α- Galactosidase	PYRase
Klebsiella pneumoniae	Gm -	**Negative** (Freydiere and Gille, 1991), (Cooke *et al*., 1999)	**Positive** (Kämpfer *et al*., 1991)	**Positive** (Inoue *et al*. 1996)
Morganella morganii	Gm -	**Negative** (Freydiere and Gille, 1991)	**Negative** (Perry *et al*.1999)	**Positive** (Inoue *et al*. 1996)
Proteus hauseri	Gm -	**Unknown**	**Unknown**	**Unknown**
Proteus mirabilis	Gm -	**Negative** (Freydiere and Gille, 1991), (Cooke *et al*., 1999)	**Negative** (Kämpfer *et al*., 1991)	**Negative** (Inoue *et al*.1996)
Proteus vulgaris	Gm -	**Negative** (Freydiere and Gille, 1991)	**Negative** (Kämpfer *et al*., 1991)	**Negative** (Inoue *et al*.1996)
Providencia rettgeri	Gm -	**Unknown**	**Unknown**	**Unknown**
Pseudomonas aeruginosa	Gm -	**Positive** (Freydiere and Gille, 1991)	**Negative** (Freydiere and Gille, 1991;Kämpfer *et al*., 1991)	**Positive** (Freydiere and Gille, 1991)
Pseudomonas otitidis	Gm -	**Unknown**	**Unknown**	**Unknown**
Pseudomonas species	Gm -	**Variable (depending on species)** (Freydiere and Gille, 1991)	**Negative** (Perry *et al*.1999)	**Variable (**depending on species**)** (Mulczyk & Szewczuk, 1970)
Serratia rubidaea	Gm -	**Unknown**	**Unknown**	**Unknown**
Serratia marcescens • (NCTC 10211)	Gm -	**Negative** (API E 20 strips; Freydiere and Gille, 1991; Cooke *et al*., 1999), **Positive** (Own work in RVS and TSB)	**Negative** (Freydiere and Gille, 1991) **Negative** (Own work, in RVS and TSB)	**Positive** (Inoue *et al*., 1996.) **Positive** (Own work in RVS and TSB)
Staphylococcus epidermidis	Gm +	**Unknown**	**Unknown**	**Negative** (De Paulis *et al*., 2003)
Streptococcus salivarius • (NCTC 8616)	Gm +	**Positive** (Kalantzopoulos *et al*., 1990) **Positive** (Own work in TSB)	**Negative** (API strips), **Negative** (Own work in TSB)	**Negative** (Panosian & Edberg, 1989), **Negative** (Own work in TSB)

8.2.2.1 Milk samples

The analysis of whole milk samples digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) showed that no bacteria isolated and no VOCs detected. The results of spiked whole milk samples (Table 8.7) is as expected, *Salmonella* species are isolated and 2-chlorophenol and phenol were detected. While in full cream milk and semi-skimmed milk samples *Hafnia alvei* were isolated and C-8 esterase activity detected as shown in Table 8.8 and Table 8.10, respectively. When these samples digested in RVS containing vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) there were no bacteria isolated and no VOCs detected. The spiked full cream milk and semi-skimmed milk samples digested in RVS shown expected result as shown in Table 8.9 and Table 8.11, respectively. A schematic diagram of digestion and incubation steps of milk (whole milk) samples with the isolated pathogens is shown in Figure 8.1.

Therefore, these milk samples results support the use of RVS as an alternative enrichment medium to BPW. As a consequence, the developed detection method of *Salmonella* in milk samples looks promising.

8.2.2.2 Cheese sample

Cheddar cheese samples digested in RVS without vancomycin (5 mg/L) and novobiocin (10 mg/L) being added in this step show no VOCs detected and no bacteria isolated. The spiked cheddar cheese samples show normal results, where *S. stanley* isolated and 2-chlorophenol and phenol were detected (Table 8.12). A schematic diagram of digestion and incubation steps of cheese samples with the isolated pathogens is shown in Figure 8.2.

Table 8.7 liberated VOCs and isolated pathogens in spiked whole milk samples digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	spiked *S. Stanley* (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	1.32	*Salmonella species*	*Salmonella species*	0.0140	0.046
		2	1.31	*Salmonella species*	*Salmonella species*		
		3	1.35	*Salmonella species*	*Salmonella species*		
α- Galactosidase	Phenol	1	20.2	*Salmonella species*	*Salmonella species*	0.0451	0.1503
		2	20.1	*Salmonella species*	*Salmonella species*		
		3	20.5	*Salmonella species*	*Salmonella species*		
PYRase	3-Fluoroaniline	1	ND	*Salmonella species*	*Salmonella species*	0.0049	0.0163
		2	ND	*Salmonella species*	*Salmonella species*		
		3	ND	*Salmonella species*	*Salmonella species*		

ND =not detected

Table 8.8 liberated VOCs and isolated pathogens in full cream milk samples digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	0.72	Hafnia alvei	Hafnia alvei	0.0140	0.046
		2	1.2	Hafnia alvei	Hafnia alvei		
		3	0.82	Hafnia alvei	Hafnia alvei		
α- Galactosidase	Phenol	1	ND	Hafnia alvei	Hafnia alvei	0.0451	0.1503
		2	ND	Hafnia alvei	Hafnia alvei		
		3	ND	Hafnia alvei	Hafnia alvei		
PYRase	3-Fluoroaniline	1	ND	Hafnia alvei	Hafnia alvei	0.0049	0.0163
		2	ND	Hafnia alvei	Hafnia alvei		
		3	ND	Hafnia alvei	Hafnia alvei		

ND = not detected

Table 8.9 liberated VOCs and isolated pathogens in spiked full cream milk samples digested in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	spiked *S. Stanley* (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	1.63	*Salmonella species*	*Salmonella species*	0.0140	0.046
		2	1.10	*Salmonella species*	*Salmonella species*		
		3	1.07	*Salmonella species*	*Salmonella species*		
α- Galactosidase	Phenol	1	9.55	*Salmonella species*	*Salmonella species*	0.0451	0.1503
		2	3.3	*Salmonella species*	*Salmonella species*		
		3	3.2	*Salmonella species*	*Salmonella species*		
PYRase	3-Fluoroaniline	1	ND	*Salmonella species*	*Salmonella species*	0.0049	0.0163
		2	ND	*Salmonella species*	*Salmonella species*		
		3	ND	*Salmonella species*	*Salmonella species*		

ND = not detected

Table 8.10 liberated VOCs and isolated pathogens in semi-skimmed milk samples digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	0.10	Hafnia alvei	Hafnia alvei	0.0140	0.046
		2	0.08	Hafnia alvei	Hafnia alvei		
		3	0.10	Hafnia alvei	Hafnia alvei		
α- Galactosidase	Phenol	1	ND	Hafnia alvei	Hafnia alvei	0.0451	0.1503
		2	ND	Hafnia alvei	Hafnia alvei		
		3	ND	Hafnia alvei	Hafnia alvei		
PYRase	3-Fluoroaniline	1	ND	Hafnia alvei	Hafnia alvei	0.0049	0.0163
		2	ND	Hafnia alvei	Hafnia alvei		
		3	ND	Hafnia alvei	Hafnia alvei		

ND = not detected

Table 8.11 liberated VOCs and isolated pathogens in spiked semi-skimmed milk samples digested in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	spiked *S. Stanley* (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	3.02	*Salmonella species*	*Salmonella species*	0.0140	0.046
		2	4.04	*Salmonella species*	*Salmonella species*		
		3	3.87	*Salmonella species*	*Salmonella species*		
α- Galactosidase	Phenol	1	13.5	*Salmonella species*	*Salmonella species*	0.0451	0.1503
		2	26.6	*Salmonella species*	*Salmonella species*		
		3	17.8	*Salmonella species*	*Salmonella species*		
PYRase	3-Fluoroaniline	1	ND	*Salmonella species*	*Salmonella species*	0.0049	0.0163
		2	ND	*Salmonella species*	*Salmonella species*		
		3	ND	*Salmonella species*	*Salmonella species*		

ND = not detected

Table 8.12 liberated VOCs and isolated pathogens in spiked cheddar cheese digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	spiked with *S. Stanley* (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	0.83	*Salmonella species*	*Salmonella species*	0.0140	0.046
		2	0.63	*Salmonella species*	*Salmonella species*		
		3	1.3	*Salmonella species*	*Salmonella species*		
α- Galactosidase	Phenol	1	29.4	*Salmonella species*	*Salmonella species*	0.0451	0.1503
		2	10.4	*Salmonella species*	*Salmonella species*		
		3	16.7	*Salmonella species*	*Salmonella species*		
PYRase	3-Fluoroaniline	1	ND	*Salmonella species*	*Salmonella species*	0.0049	0.0163
		2	ND	*Salmonella species*	*Salmonella species*		
		3	ND	*Salmonella species*	*Salmonella species*		

ND = not detected

Other types of cheeses digested in RVS with and without addition of the antibiotics show no improvement on the specificity of the detection method. In detail, the goat milk cheese samples result shows detection of C-8 esterase activities and isolation to *Streptococcus salivarius* when no antibiotics were added in the enrichment step (Table 8.13). Goat milk cheese samples were further tested after addition of vancomycin (5 mg/L) and novobiocin (10 mg/L). Unfortunately, detection of the same VOCs and isolation of the same bacteria was observed (Table 8. 14).

Analysis of Claxstone blue cheese samples digested in RVS broth without addition of vancomycin and novobiocin showed detection to the VOCs along with isolation of the pathogenic *Raoultella ornithinolytica* in all the samples (Table 8.15). While the VOCs and *Lactobacillus rhamnosus* were detected in the same samples digested in RVS with addition of the antibiotics (Table 8.16). *Raoultella ornithinolyitca* is a Gram negative bacteria, formerly named *Klebsiella ornithinolytica* (Walckenaer *et al*., 2004), and has been isolated from raw cow's milk samples (Lazzi *et al*., 2014). Human infection with *Raoultella ornithinolytica* is rare with only a few cases of urinary tract infection having been reported previously (Nakasone *et al*., 2015). Isolation of *Lactobacillus rhamnosus* is also possible in cheese samples *as* these species are encountered in many dairy products, where they can be added as a probiotic microorganisms or can be naturally present arising from raw milk and play a significant role during cheese ripening, leading to the formation of flavor (Lazzi *et al*., 2014). In addition, *Lactobacillus rhamnosus* can also survive food processing and persist in finished products (Comunian *et al*., 2010). However, *Lactobacilli* also cause some human diseases and have been identified as potential emerging pathogens in elderly and immunocompromised patients (Harty *et al*., 1994).

Table 8.13 liberated VOCs and isolated pathogens in goat milk cheese digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	1.36	*Streptococcus salivarius*	NG	0.0140	0.046
		2	1.35	*Streptococcus salivarius*	NG		
		3	1.47	*Streptococcus salivarius*	NG		
α- Galactosidase	Phenol	1	ND	*Streptococcus salivarius*	NG	0.0451	0.1503
		2	ND	*Streptococcus salivarius*	NG		
		3	ND	*Streptococcus salivarius*	NG		
PYRase	3-Fluoroaniline	1	ND	*Streptococcus salivarius*	NG	0.0049	0.0163
		2	ND	*Streptococcus salivarius*	NG		
		3	ND	*Streptococcus salivarius*	NG		

ND = not detected; NG = no growth

Table 8.14 liberated VOCs and isolated pathogens in goat milk cheese digested in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Un-spiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	0.94	*Streptococcus salivarius*	NG	0.0140	0.046
		2	0.84	*Streptococcus salivarius*	NG		
		3	1.04	*Streptococcus salivarius*	NG		
α- Galactosidase	Phenol	1	ND	*Streptococcus salivarius*	NG	0.0451	0.1503
		2	ND	*Streptococcus salivarius*	NG		
		3	ND	*Streptococcus salivarius*	NG		
PYRase	3-Fluoroaniline	1	ND	*Streptococcus salivarius*	NG	0.0049	0.0163
		2	ND	*Streptococcus salivarius*	NG		
		3	ND	*Streptococcus salivarius*	NG		

ND = not detected; NG = no growth

Table 8.15 liberated VOCs and isolated pathogens in Claxstone blue cheese digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Unspiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	14.0	*Raoultella ornithinolytica*	*Raoultella ornithinolytica,*	0.0140	0.046
		2	15.3	*Raoultella ornithinolytica*	*Raoultella ornithinolytica,*		
		3	12.2	*Raoultella ornithinolytica*	*Raoultella ornithinolytica,*		
α- Galactosidase	Phenol	1	27.2	*Raoultella ornithinolytica,*	*Raoultella ornithinolytica,*	0.0451	0.1503
		2	26.3	*Raoultella ornithinolytica,*	*Raoultella ornithinolytica,*		
		3	24.7	*Raoultella ornithinolytica,*	*Raoultella ornithinolytica,*		
PYRase	3-Fluoroaniline	1	0.81	*Raoultella ornithinolytica*	*Raoultella ornithinolytica,*	0.0049	0.0163
		2	0.63	*Raoultella ornithinolytica,*	*Raoultella ornithinolytica,*		
		3	0.53	*Raoultella ornithinolytica,*	*Raoultella ornithinolytica,*		

Table 8.16 liberated VOCs and isolated pathogens in Claxstone blue cheese digested in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Unspiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	6.17	*Lactobacillus rhamnosus*	NG	0.0140	0.046
		2	6.22	*Lactobacillus rhamnosus*	NG		
		3	4.40	*Lactobacillus rhamnosus*	NG		
α- Galactosidase	Phenol	1	24.2	*Lactobacillus rhamnosus*	NG	0.0451	0.1503
		2	23.9	*Lactobacillus rhamnosus*	NG		
		3	25.9	*Lactobacillus rhamnosus*	NG		
PYRase	3-Fluoroaniline	1	0.04	*Lactobacillus rhamnosus*	NG	0.0049	0.0163
		2	0.03	*Lactobacillus rhamnosus*	NG		
		3	≥ LOQ 0.01	*Lactobacillus rhamnosus*	NG		

≥ LOQ = below quantification limit, NG = no growth

In the studied Bassett Stilton cheese samples when the samples were digested in RVS without addition of vancomycin and novobiocin all the VOCs were detected and *Enterococcus faecalis* were the only bacteria isolated (Table 8.17). However, when the samples digested in presence of the antibiotics the false positive results were detected (Table 8.18).

The results of MALDI-TOF-MS identification to the isolated bacteria in the tested Bassett Stilton cheese samples came out with presence of *Enterococcus faecalis.* However, Enterococcus faecalis were reported as PYRase positive and α- galactosidase negative (Table 8.6). Therefore, the detected α- galactosidase activity in Bassett Stilton cheese samples must be related to other bacteria that could not be isolated on CLED agar plates in 24 h incubation at 37 ºC. And the absence of the PYRase activity of the isolated *Enterococcus faecalis* could be due to the undetectable signal of 3-fluoroanaline or may be PYRase and α-galactosidase are variable and depends on the species of *Enterococcus faecalis*.

The most obvious finding to emerge from these experiments is that, the developed detection method of *Salmonella* is working properly in milk samples and cheddar cheese samples. However, it is unfortunate that the method specificity when applied to other types of cheese is still of concern. Therefore, further experimental investigations are needed.

8.3 Evaluation of some other parameters

The false positive results detected in Bassett Stilton cheese samples and the isolation of bacteria in cheese samples after selective enrichment process give rise to the possibility that the large inoculated volume or/and the long inoculation time are the reasons for the presence of these isolates in the tested samples. Therefore, investigations to these parameters are needed.

Table 8.17 liberated VOCs and isolated pathogens in Bassett stilton cheese digested in RVS and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Unspiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	16.5	*Enterococcus faecalis*	NG	0.0140	0.046
		2	9.87	*Enterococcus faecalis*	NG		
		3	16.0	*Enterococcus faecalis*	NG		
α- Galactosidase	Phenol	1	20.2	*Enterococcus faecalis*	NG	0.0451	0.1503
		2	22.0	*Enterococcus faecalis*	NG		
		3	17.2	*Enterococcus faecalis*	NG		
PYRase	3-Fluoroaniline	1	0.23	*Enterococcus faecalis*	NG	0.0049	0.0163
		2	0.28	*Enterococcus faecalis*	NG		
		3	0.22	*Enterococcus faecalis*	NG		

NG = no growth

Table 8.18 liberated VOCs and isolated pathogens in Bassett stilton cheese digested in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) and incubated in RVS with vancomycin (5 mg/L) and novobiocin (10 mg/L) for 24 hours

Enzyme	Label	Individual results	Unspiked (µg/mL)	Isolates on CLED	Isolates on ABC	LOD (µg/mL)	LOQ (µg/mL)
C-8 esterase	2-Chlorophenol	1	19.1	*Enterococcus faecalis*	NG	0.0140	0.046
		2	12.3	*Enterococcus faecalis*	NG		
		3	13.2	*Enterococcus faecalis*	NG		
α- Galactosidase	Phenol	1	25.3	*Enterococcus faecalis*	NG	0.0451	0.1503
		2	22.0	*Enterococcus faecalis*	NG		
		3	18.5	*Enterococcus faecalis*	NG		
PYRase	3-Fluoroaniline	1	ND	*Enterococcus faecalis*	NG	0.0049	0.0163
		2	ND	*Enterococcus faecalis*	NG		
		3	ND	*Enterococcus faecalis*	NG		

ND = no detection; NG = no growth

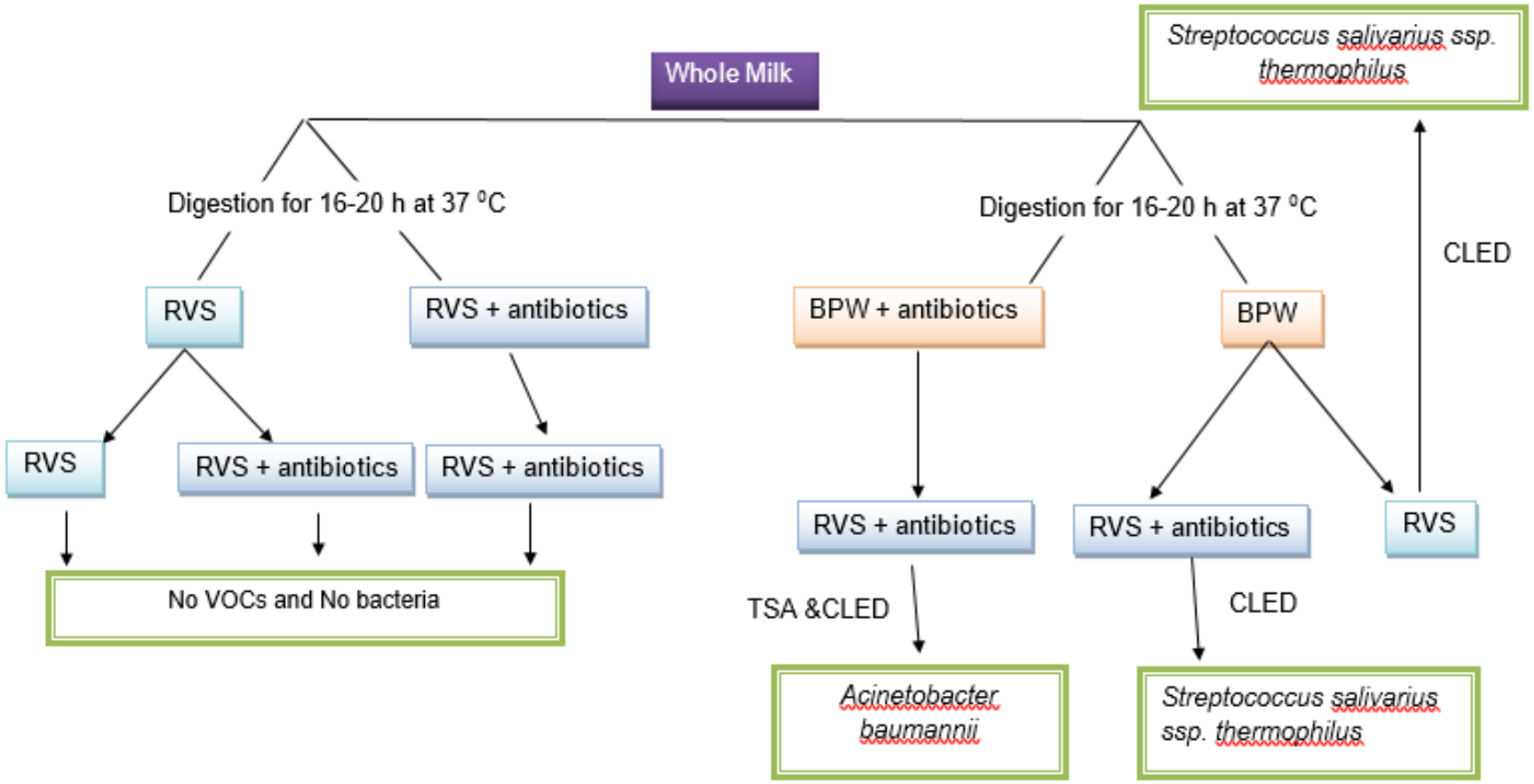


Figure 8.1 Schematic diagram of milk samples digestion and incubation steps with the isolated pathogens

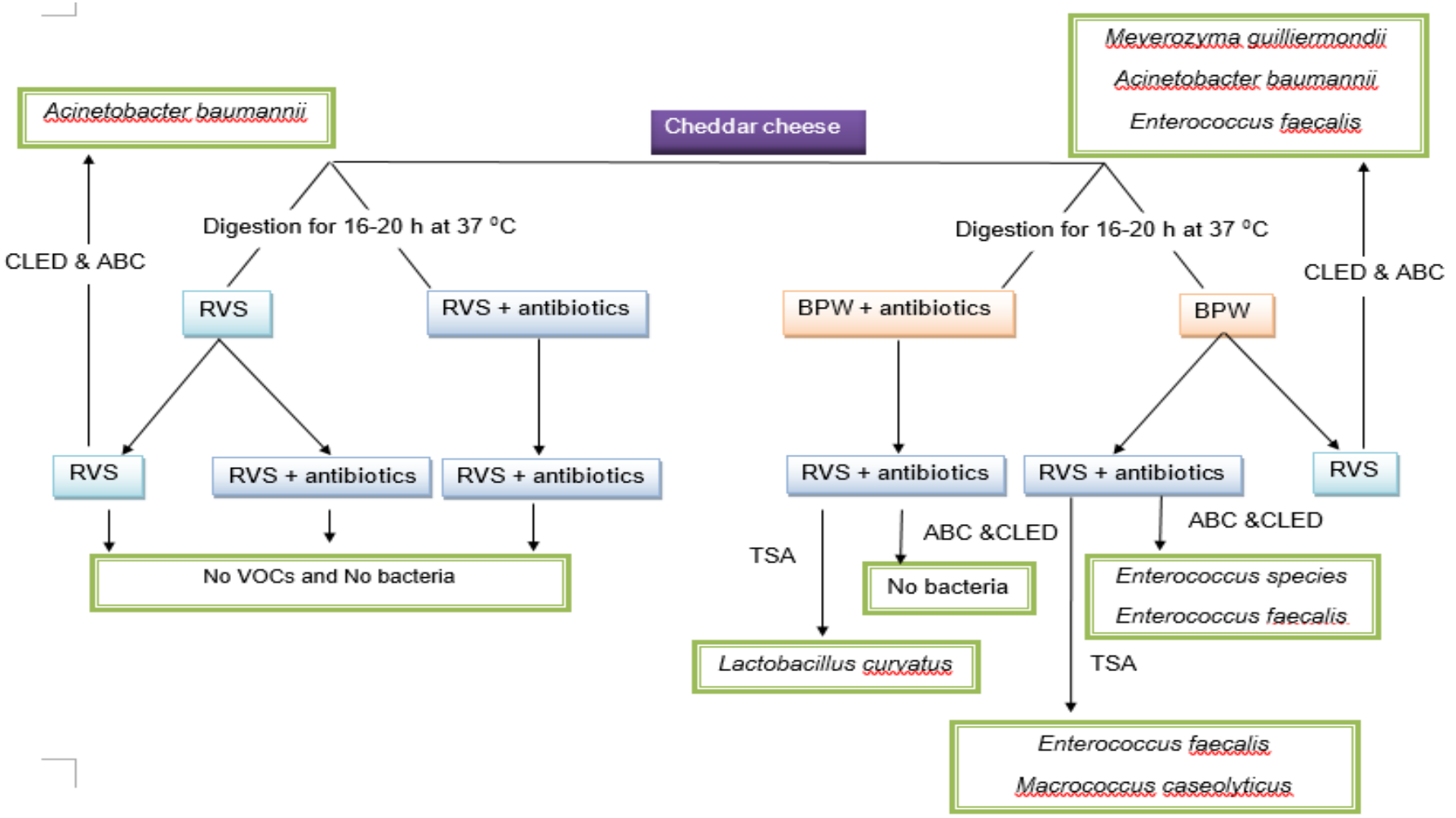


Figure 8.2 Schematic diagram of cheese samples digestion and incubation steps with the isolated pathogens

8.3.1 Inoculation volume

In the developed *Salmonella* detection method applied to food samples, before the analysis, the enrichment step (digestion step) always followed by inoculation and incubation of 1 mL enriched food sample in 10 mL RVS broth for 18-24 h at 37 ºC. It may be the case therefore that by inoculation of 1 mL of enriched food is heavily loading organisms into next step (incubation in selective broth) causes presence of the pathogens in the samples and detection of the enzyme activities. Further studies, which take this variable into account, were carried out. The inoculated volume of cheese samples was reduced 10 times in order to remove the inoculum effect (IE). Unfortunately, the results of analysis of 0.1 mL enriched sample (in RVS with and without vancomycin 5 mg/L and novobiocin 10 mg/L) of both types of cheeses (Claxstone blue and Bassett Stilton) showed no deference in the detected VOCs and isolated bacteria with the previous obtained result when using 1 mL of the enriched sample. The false positive results were detected in both cheese samples enriched in RVS with the antibiotics. The slow growth of the bacteria enriched with the antibiotics was observed where the *Lactobacillus rhamnosus* was isolated in Claxstone blue cheese after 48 h incubation at 37 ºC.

8.3.2 Inoculation time

A 16-20 h pre-enrichment period was recommended by the standard method (ISO 6579:2002) that was subseqently applied in this study. This long time of digestion and incubation at 37 ºC may be is the variable that causes the overgrowth of the pathogens in the food samples. Therefore, reducing this time may result in better VOC analysis of the studied foods. So, the 4 h pre-enrichment period was chosen to test the cheese samples in an attempt to eliminate any false positive. The pre-enrichment proceeded in RVS with and

without antibiotics. Goat milk cheese, Claxstone blue cheese and Bassett stilton cheese samples were digested in RVS with and without vancomycin (5 mg/L) and novobiocin (10 mg/L) for 4 hours at 37 ºC. The results of goat milk cheese samples are showed detection of C-8 esterase activity (0.7 ± 0.09 µg/mL of 2-chlorophenol) and isolation of *Streptococcus salivarius ssp. thermophilus*. Claxstone blue cheese results show detection of C-8 esterase activity (5.5 ± 1.2 µg/mL of 2-chlorophenol) and α- galagtosidase (23.7 ± 4.2 µg/mL of phenol) and isolation of *Lactobacillus rhamnosus.* In Bassett stilton cheese *Enterococcus faecalis* was isolated with detection of C-8 esterase activity (8.8 ± 0.74 µg/mL of 2-chlorophenol) and α- galactosidase (8.3 ± 0.10 µg/mL of phenol). These results are similar to those obtained in 16 h enrichment period of time and presented in Tables 8.14, 8.16 and 8.18. These findings were unexpected and suggest that neither the inoculation volume nor the inoculation time cause the presence and isolation of these species in the cheese samples. It was concluded that *Enterococcus faecalis*, *Lactobacillus rhamnosus* and *Streptococcus salivarius ssp. thermophilus* are vancomycin (5 mg/L) and novobiocin (10 mg/L) resistant. This conclusion was supported by previous research where 63 *Lactobacillus rhamnosus* strains isolated from cheese samples reported to be resistant to vancomycin (Coppola *et al*., 2005). This is also supported by the study that discovered three of 170 different *E. faecalis* isolates reported to be resistant to vancomycin (32-64 µg / mL) (Sahm *et al*., 1989). However, isolation of *Streptococcus salivarius ssp. Thermophilus* is contrary to previous study which reported the susceptibility of *Streptococcus salivarius ssp. Thermophilus* isolated from probiotic products to vancomycin (Blandino *et al*., 2008). Due to the liberation of VOCs (2-chlorophenol and phenol) by these species it would not be possible to determine whether VOCs generated in an unknown cheese sample

indicates *Salmonella* contaminated cheese. The inhibition of false positive VOC signals would require further investigations into alternative combinations of selective agents to suppress the growth of VOC generating species. It can thus be suggested to add to vancomycin and novobiocin other inhibitors such as, erythromycin and lithium chloride.

8.4 Analysis of cheese samples with erythromycin and lithium chloride

Erythromycin is an antibiotic belonging to a group of drugs called macrolide antibiotics. Macrolide antibiotics slow the growth of, or sometimes kill, sensitive bacteria by reducing the production of important proteins needed by the bacteria to survive (https://www.drugs.com/search.php?searchterm=Erythromycin+). Erythromycin was chosen to inhibit *Lactobacillus rhamnosus* strains and *Streptococcus salivarius ssp. thermophilus as* the susceptibility of 15 strains of *Lactobacillus spp.*, 5 *Streptococcus salivarius ssp. thermophilus* was previously reported (Blandino *et al*., 2008; Pisano *et al*., 2014). In addition, the use of the selective agent lithium chloride (LiCl) in bacterial media to inhibit the growth of some bacteria has been previously reported (Smidt and Vidaver, 1986; Lapierre *et al*., 1992). More specifically, the inhibition of background microflora from foods and to improve selective isolation of pathogens such as, *Listeria monocytogenes* and *Staphyloococcus aureus* from food samples by lithium chloride has been previously reported (Cox *et al*., 1990). The background microflora counts decreased dramatically as the LiCl concentration increased (Cox *et al*., 1990; Mendonca and Knabel, 1994). The application of typical MICs of lithium chloride 15 g / L and erythromycin 0.75 mg / L were used in further analysis of cheese samples. These concentrations were the result of a personal communication (with John Perry, Freeman Hospital).

The combination of vancomycin (10 mg/L), novobiocin (10 mg/L), erythromycin (0.75 mg/L) and lithium chloride (15 g/L) was applied in the developed enrichment procedure (Section 8.2.2) for detection of *Salmonella* in cheese samples to inhibit the false positive results. Unfortunately, these investigations did not show any increase in the specificity of the method as no inhibitory effect for this combination on any of the targeted organisms. The results of the liberated VOCs and the isolated bacteria are shown in Table 8.19. *Lactobacillus rhamnosus* isolated from Claxstone blue cheese samples reported to be positive C-8 esterase and α-galagtosidase as obsorved in this experiment (Pisano *et al*., 2014). Figure 8.3 shows the chromatograms of the liberated VOCs by the isolated bacteria. It seems that the growth rate was reduced for all species in the presence of this combination including *S. stanley*. The bacteria isolated from cheese samples required 48 h to grow on CLED agar plates at 37 ºC. As no observed effect was detected on *Salmonella* growth when vancomycin and novobiocin were used, and *Salmonella* was known to be resistant to erythromycin (Braoudaki and Hilton, 2005) therefore, the effect of LiCl (15 g/L) on *Salmonella* was tested. The result showed suppression in the growth of *S. stanley* which was observed as the decrease of produced enzymes, for example, α- galagtosidase. The concentration of phenol liberated by *S. stanley* after overnight incubation at 37 °C without addition of LiCl (15 g/L) was 39.5 µg/mL, and was 1.13 µg/mL when LiCl was added. The phenol signal is shown in the chromatogram in Figure 8.4. The analysis of blue cheese samples using the developed *Salmonella* detection method had shown poor specificity, as the false positive results were detected. Therefore, the present results were not very encouraging to continue testing food samples without investigating the susceptibility of isolated bacteria to the used

antibiotics. Therefore, first the susceptibility of isolated bacteria to vancomycin was carried out.

Table 8.19 liberated VOCs and isolated pathogens in cheese samples digested and incubated in RVS with vancomycin (10 mg/L), novobiocin (10 mg/L), LiCl (15 g / mL) and erthromycin(0.75 mg /mL)

Cheese type	Pathogens isolates on CLED (48 h)	C-8 esterase (µg/mL)	α- galagtosidase (µg/mL)
Goat milk	*Streptococcus salivarius ssp. thermophilus*	0.6 ± 0.2	ND
Bassett Stilton	*Enterococcus faecalis*	17.2 ± 0.3	10.5 ± 1.5
Claxstone blue	*Lactobacillus rhamnosus*	3.4 ± 1.9	24.7 ± 4.9

8.5 Vancomycin susceptibility

Mannu *et al*. (2003) reported the different resistance pattern to antibiotics of pathogens isolated from food and from infected hospitalised patients. Therefore, the vancomycin sensitivity was studied on the isolated strains from the same studied cheese samples. The minimum inhibitory concentration (MIC) test of (5 x 10^5 CFU/mL) *Streptococcus salivarius ssp. thermophilus*, *Lactobacillus rhamnosus* and *Enterococcus faecalis* isolated from cheese samples was performed using the M.I.C. Evaluator™ (M.I.C.E.™) strips. The inoculated plates were incubated overnight at 37 ºC after the strips were applied. The results of the susceptibility (Figure 8.5) as the lowest concentration of vancomycin at which the strains are inhibited was found to be 0.5 mg/L to *Streptococcus salivarius ssp. Thermophilus,* > 256 mg/L to *Lactobacillus rhamnosus* and 2 mg/L to *Enterococcus faecalis.* The results of vancomycin susceptibility testing explain the isolation of *Streptococcus salivarius ssp. Thermophilus* from goat milk cheese samples, as it is clear that the MIC of this strain is higher than the concentration applied in the cheeses samples analysis. In addition, it is obvious that the detected false positive result in Bassett Stilton cheese samples should not be

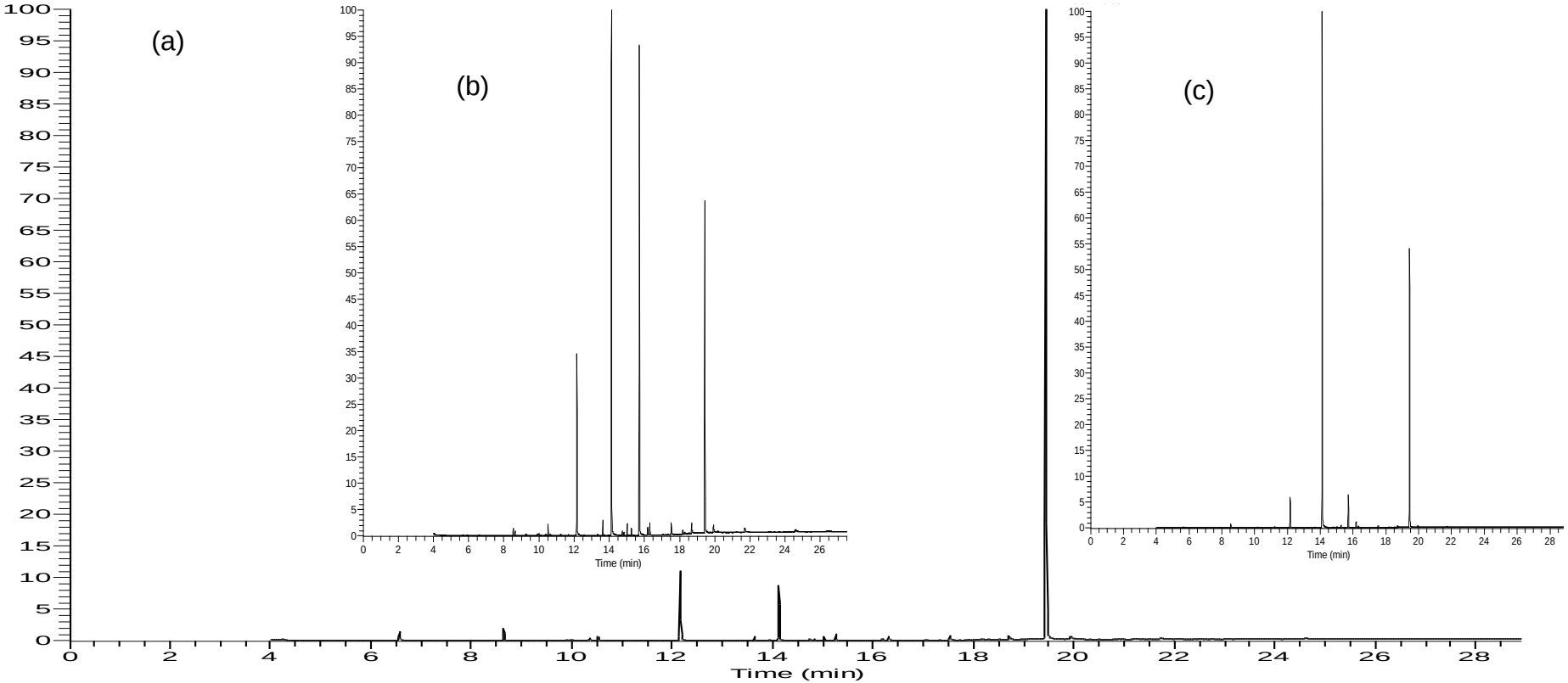


Figure 8.3 Chromatogram showing the VOCs liberated by (a) *Streptococcus salivarius ssp. Thermophilus*, (b) *Lactobacillus rhamnosus*, (c) *Enterococcus faecalis* isolated from cheese samples

(1 mL) digested and incubated overnight at 37 ºC in RVS with vancomycin (10 mg/L), novobiocin (10 mg/L), lithium chloride (15 g/L) and erythromycin (0.75 mg/L) and analyzed with non-polar GC column and polar SPME fiber; NMP (t_R = 12.3 min), 2-chlorophenol (t_R 14.1 min), phenol (t_R 15.7 min) and 2-chlorophenyl octanoate (t_R 19.5 min). Other peaks are either unknown compounds from the broth or background noise from the SPME fiber

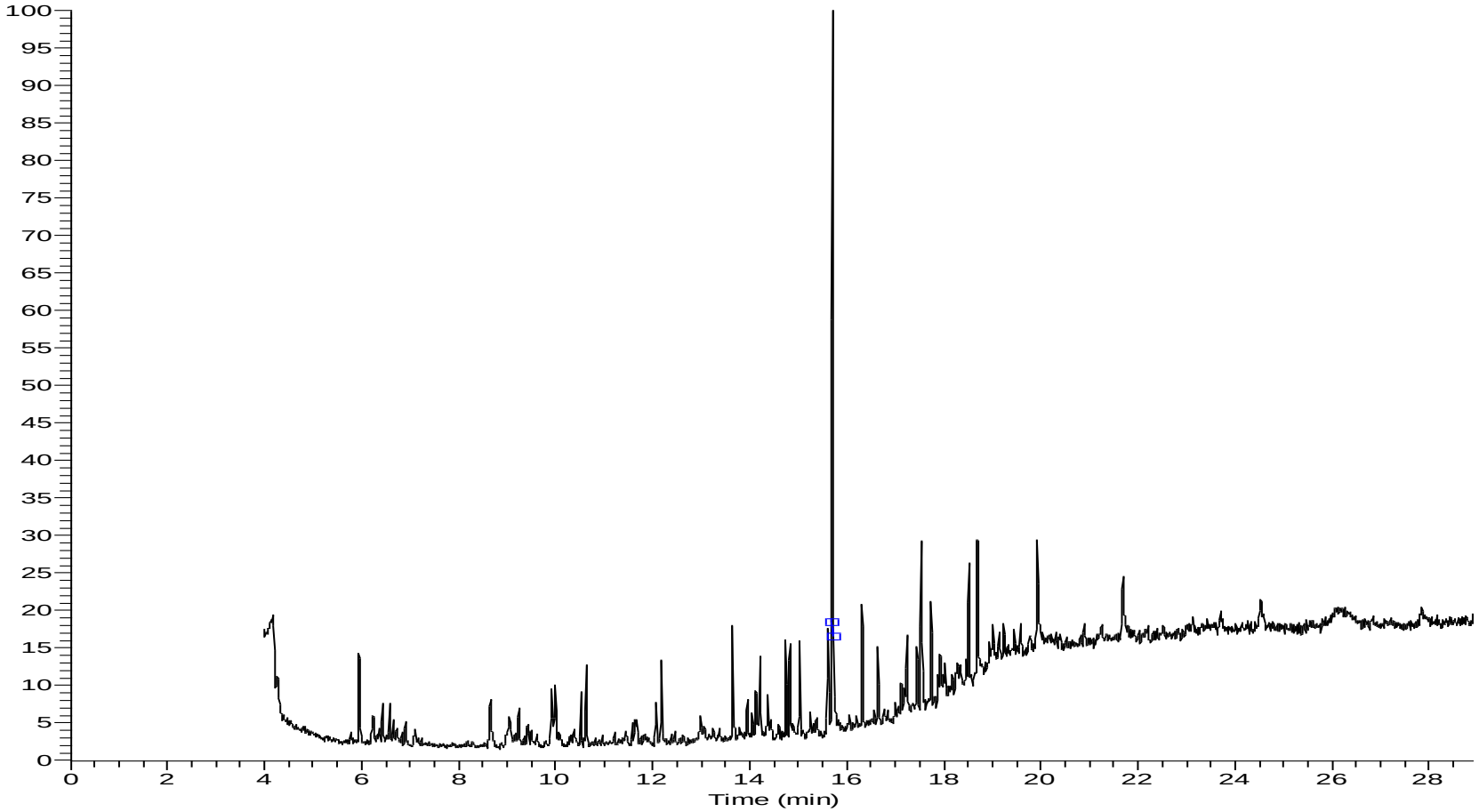


Figure 8.4 Chromatogram showing phenol (1.13 µg/mL) @ 15.7 min liberated by *S. stanley* incubated overnight at 37 °C in RVS with 15 g /L lithium chloride. Other peaks are either unknown compounds from the broth or background noise from the SPME fiber

detected as *Enterococcus faecalis* were susceptible to 2 mg/L vancomycin which is lower than the one used with the analysis experiments (10 mg/L).

The vancomycin certainly failed and it is difficult to explain this result, but it might be related to either due to the adverse impact of the broth, effect of the food or excessively high inoculum of bacteria. The vancomycin susceptibility results are contrary to that of Mathur and Singh, (2005) who detected vancomycin resistance of species of *Enterococci* and *Lactobacillus* isolated from fermented milk products. However, this study (Mathur and Singh, 2005) is supporting our finding in cheese samples.

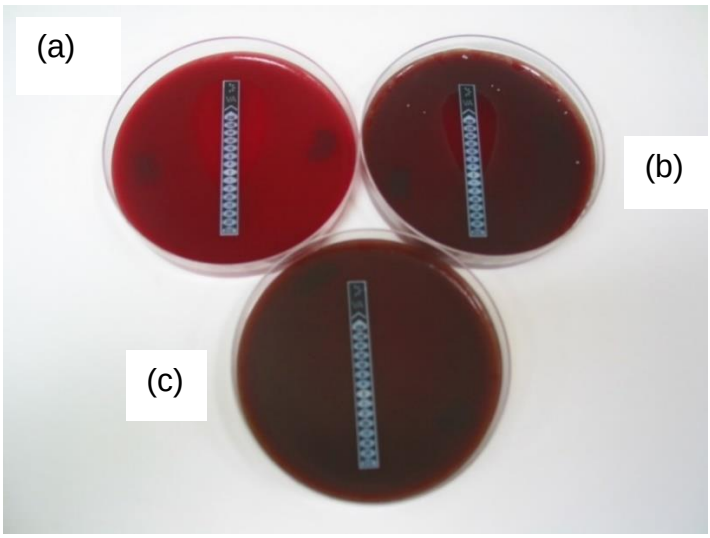


Figure 8.5 Vancomycin susceptibility of isolated strains using M.I.C. Evaluator strips (a) *Lactobacillus rhamnosus,* (b) Enterococcus *faecalis and* (c) *Streptococcus salivarius ssp. Thermophilus*

8.6 Summary and future work

The main goal of this chapter was to improve the specificity of *Salmonella* detection method. This chapter has discussed the results of VOCs analysis of milk and cheese samples after pre-enrichment process in various media with and without addition of antibiotics. The use of *Salmonella* selective media (RVS) in

pre- enrichment step has given better specificity than non-selective medium (BPW) to the detection method.

The investigations of milk and cheddar cheese samples demonstrate that the developed VOC method could potentially be used to detect *Salmonella* contamination in milk and cheddar cheese. However, due to the liberation of VOCs (2-chlorophenol and phenol) by antimicrobial-resistant pathogens in cheese samples it would not be possible to determine whether VOCs generated in an unknown cheese sample indicates *Salmonella* contaminated cheese.

The inhibition of false positive VOC signals failed by using a combination of selective agents (vancomycin, novobiocin, erythromycin and lithium chloride). The strains *Streptococcus salivarius ssp. Thermophilus*, *Lactobacillus rhamnosus* and *Enterococcus faecalis* are susceptible to vancomycin in pure culture, however in cheese samples were vancomycin resistance.

Streptococcus salivarius ssp. Thermophilus, *Lactobacillus rhamnosus* and *Enterococcus faecalis* isolated from cheese samples could be present in the cheese sample due to their use as probiotics microorganisms (Yerlikaya, 2014). They are known to be safe for most people and they have to be safe to consume and show no antibiotic resistance (Yerlikaya, 2014). However, it's worth noting that they resist the effect of the combination of vancomycin (10 mg / L), novobiocin (10 mg /L), erythromycin (0.75 mg / L) and lithium chloride (15 g / L). Also, it's worth noting that they have effects the specificity of *Salmonella* detection method as they produce false positive results.

Although the results of milk samples analysis are based on a small sample type, the findings suggest that the VOC analysis method could be used to develop an easy, simple and efficient system for detection of *Salmonella* in food samples.

Therefore, the next suggested future step will be evaluation of VOCs liberated from *Salmonella* enzyme substrates to develop an optical detection method. For example, colorimetric sensors where one can observe with the naked eye the presence of pathogenic microorganisms in the sample through a colour change without the need for any analytical instrument. This would require further investigations.

Chapter 9: Conclusions and future work

9.1 Conclusion

Salmonella is an important pathogen (bacteria) that commonly causes foodborne illness. One important research topic on food safety is pathogen detection. In these investigations, the main goal was to develop a rapid, sensitive and selective system for the detection of *Salmonella* in food samples. This was achieved by analysis of the liberated *Salmonella* VOCs using HS-SPME-GC/MS. The entire VOC profiles of *Salmonella* VOCs using 6 strains of *Salmonella* inoculated in sterile BHI, TSB and RVS broths were determined. The most important compounds detected were alcohol, ester and ketone compounds. However, the VOCs profile of *Salmonella* strains cannot be used as a marker for the presence or absence of *Salmonella* in food samples as they exist naturally.

A specific liberated VOC that acts as a marker to be employed as a tool for the contaminated food was needed. This was done by the use of enzyme substrate reactions. This study highlights the potential of designing enzyme substrates to liberate exogenous VOCs for *Salmonella* identification. It is unlikely that a single VOC could act as a marker for a specific bacterial species. Therefore, it was important to test sufficient enzymes of *Salmonella* to develop unambiguous identification in food samples. The successfully chosen key enzymes of *Salmonella* were α-galactosidase, C-8 esterase and pyrrolidonyl peptidase (PYRase). The VOC analysis was carried out after additional of enzyme substrates (to the growth media) that interact with *Salmonella* enzymes to generate unique biomarkers. The investigation of commercial and synthesised enzyme substrates ended up with an assay working for *Salmonella* detection and identification in food samples through monitoring VOCs generated by *Salmonella* during hydrolysis of the specific substrates.

The results of this investigation found that all *Salmonella* strains hydrolysed the substrate phenyl α-D-galactopyranoside and generated phenol as a marker of α-galactosidase activity, and hydrolysed the synthesised enzyme substrate 2-chlorophenyl octanoate and liberated 2-chlorophenol as a marker of C-8 esterase activity. While the absence of 3-fluoroanaline as a result of the synthesised enzyme substrate L-pyrrollidonyl fluoroanilide not being hydrolysed was a useful indicator for *Salmonella* presence. The developed approach shows potential for future application in food samples to detect and identify *Salmonella* species in food samples of a level as low as 10^0 CFU /mL within a 5 h incubation at 37 ºC by the detection of the liberated VOCs.

The developed VOC method was applied to identify *Salmonella* in food types considered as the most common sources of *Salmonella* (milk, cheese, raw eggs, and raw chicken). All food samples were *Salmonella* free. The method was successful in identifying *Salmonella* in spiked samples based on the detection of the expected VOCs, however a lack of specificity was evident. False positives were detected as phenol and 2-chlorophenol signals in the studied samples, due to the presence of other bacteria (pathogenic) in food samples. Such bacteria are *Pseudomonas aeruginosa, Escherichia coli, Proteus haauseri, Enterococcus faecalis, Streptococcus salivarius.* An important finding from these experiments was that inhibition of interfering pathogens was essential. Inhibition of some of these pathogens was achieved with the addition of the specific antibiotics vancomycin (5 mg / L) and of novobiocin (10 mg / L). However, the complicated matrices and the heavy background microflora in food samples allowed the production of false positive signals from cleavage of enzyme substrates by resistant pathogens in the tested milk and cheese samples. An investigation of the use of a selective pre-enrichment media (RVS) results in a

successful detection method for this approach and detect *Salmonella* contamination on milk samples and cheddar cheese samples. However, failed in detection of *Salmonella* in other cheese samples (especially blue cheese type).

Another combination of selective agents (vancomycin 10 mg/L, novobiocin 10 mg/L, erythromycin 0.75 mg/L and lithium chloride 15 g/L) along with using a selective pre-enrichment media RVS was applied to analyse cheese samples in order to improve the specificity of the method and suppresses the pathogens producing the false positive. Unfortunately, the results obtained did not completely suppress the pathogens. Although the method was successful in detecting *Salmonella* in milk and cheddar cheese samples, it was concluded that, to some extent incomplete specificity of the *Salmonella* detection method (specially for cheese samples) was evident.

9.2 Future work

The analytical method HS-SPME GC/MS has shown a potential detection to *Salmonella* in milk samples and cheddar cheese samples. However, the specificity of the developed assay for detection of *Salmonella* in cheese samples is still the main issue in the current project and improvements are continuing. The study should be repeated using samples of food that are important sources of *Salmonella* infection. For example, unpasteurized fruit juices, mayonnaise, ice cream, pre-packaged salad products, cucumbers and raw seed sprouts. More studies are required to investigate the possible useful antibiotics and the species of pathogenic bacteria would help suppress. Inhibition of antibiotic resistant *Streptococcus salivarius ssp. Thermophilus*, *Lactobacillus rhamnosus* and *Enterococcus faecalis* would help in improve the method specificity in cheese analysis.

Further work needs to be done to establish the inhibition of these isolates. This could be done by using different types of selective agents such as; antibiotics or bacteriocins. Bacteriocins, are antimicrobial peptides produced by certain bacteria. These molecules exhibit significant potency against other bacteria (including antibiotic-resistant strains) and used as alternatives to traditional antibiotics (Cotter *et al*., 2013). Bacteriocins have been known for approximately 90 years and have been described for the genera *Lactobacillus*, *Enterococcus and Staphylococcus (*Altuntas, 2013). Antibiotics and bacteriocins need to be selected based on the isolated species and relevant literature. Further studies need to be carried out in order to determine the optimum concentrations for each specific application and organism of interest. Further experimental investigations could be applied to cheese samples to completely suppress pathogens and all related species, thereby emanating all false positive VOCs signals.

Detecting food-borne pathogens as quickly and reliably as possible is a matter of public safety. The challenge for the future is to detect the food contamination in the very early stages of the food processing. The analysis of liberated VOCs by HS-SPME GC/MS requires complicated assay steps and time-consuming and costly techniques and need high technical assistance. Therefore, alternative approach for the detection of the emitted VOC from enzyme substrates are needed for the rapid, accurate and simple detection of pathogens in foods. Biosensors hold great promise for addressing the analytical needs in practical pathogen detection.

Among these, optical sensors, especially colorimetric sensors, allow easy-to-use, rapid (within 15 min), portable, and cost-effective detection (Tait *et al*., 2015; Yoo and Lee, 2016). In colorimetric biosensors system one can easily and instantly observe with the naked eye the presence of pathogenic microorganisms

in the sample through a colour change without the need for any analytical instrument.

It is recommended that further research be undertaken to investigate the detection of *Salmonella* in the sample by observing the generated colour when the enzyme associated with *Salmonella* reacts with the inoculated substrate to produce a VOC. This VOC could be trapped in the headspace of the sample into a matrix (e.g. agarose gel) containing a colorimetric reagent. Agarose gel has been reported to be a suitable VOC trapping matrix and host for the colour-generating reagents (Tait *et al*., 2015). The proof of concept can be demonstrated using the commercially available phenyl α-D-galactopyranoside which liberates phenol in the presence of bacteria with α-galactosidase activity, a synthesised substrate, L-pyrrollidonyl fluoroanilide which liberates 3-fluoroanaline in the presence of bacteria with PYRase activity and the synthesised 2-chlorophenyl octanoate which liberates 2-chlorophenol in the presence of bacteria with C-8 esterase activity.

Phenol and 2-chlorophenol can be optically detected by their reaction with 4-aminoantipyrine which is a sensitive reagent for detecting phenols giving a red-violet colour (Ettinger *et al*., 1951) whereas the 3-fluoroanaline can be optically detected by its reaction with sodium 1,2-naphthoquinone-4-sulfonate (NQS) giving a “baby” pink colour (Feigl, 1961; Li and Yang, 2007) or using 3-methyl-2-benzothiazolone hydrazine (Sawicki *et al*., 1961).

The developed colour could be optically detected by either the naked eye or colorimetric analysis. The colorimetric analysis allows quantification of the liberated exogenous VOC by determining the maximum absorption wavelength of the reaction product and measure the absorbance at this wavelength.

More development of a colorimetric method that would include the use of a specific indicator or dye impregnated onto a support/dipstick is possible. By suspending the device in the headspace above the food sample would allow detection of the unique VOC by a color change in the indicator or dye. As this proposal work displays potential for the development simple and low-cost detection devices for detecting bacteria in clinical and food samples the requirement for HS-SPME-GC/MS could be negated. The advantages the optical detection method can be used to develop more advanced prototype sensor which is more suitable in food industry.

Chapter 10: References

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